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(71) Applicants: CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US). AGRACETUS [US/US]; 8520 University Green, Middleton, WI 53562 (US).

(72) Inventors: GELFAND, David, H. ; 6208 Chelton Drive, Oakland, CA 94611 (US). BARTON, Kenneth, A. ; 1718 Aurora Street, Middleton, WI 53562 (US).

(74) Agent: HALLUIN, Albert, P.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US).

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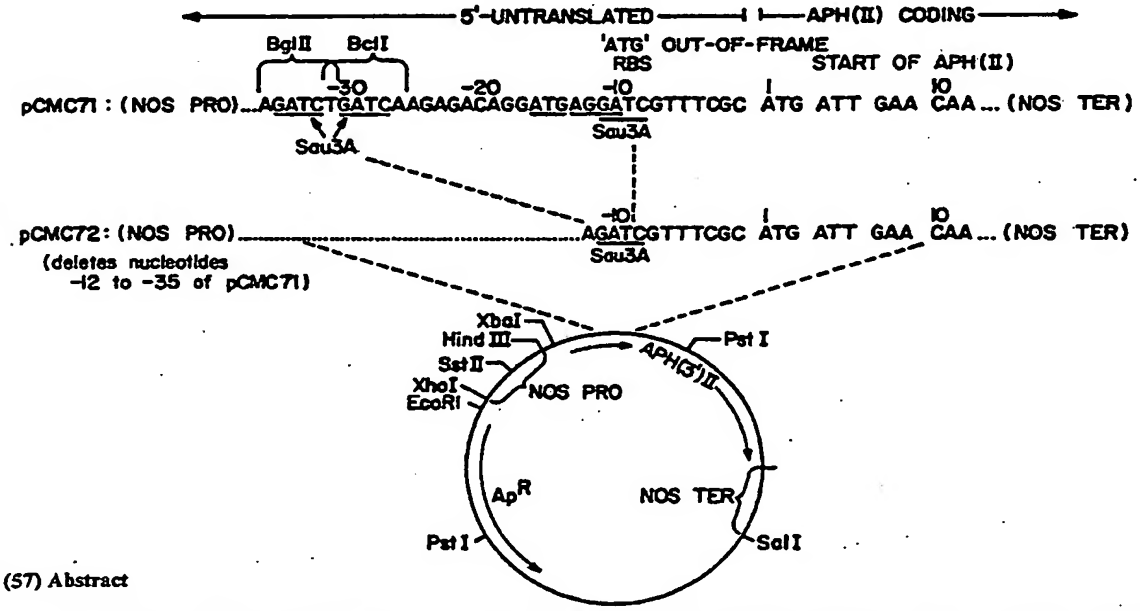

(57) Abstract

Vectors and methods suitable for both direct and *Agrobacterium*-mediated transformation of plants. The vectors comprise easily manipulated expression cassettes and optionally contain border sequences associated with *Agrobacterium* plasmid DNA transfer. By using an intermediate carrier vector which contains the requisite expression control sequences in accessible form, a wide variety of single and multiple cassette vectors can be constructed.

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METHODS AND VECTORS FOR TRANSFORMATION OF PLANT CELLS

Technical Field

The present invention relates to the field of application of recombinant DNA techniques to the transformation of higher plants. More specifically, the invention relates to vectors useful in transforming higher plants and in conferring desired properties on them, and to methods of employing such vectors in transformations.

Background Art

After nearly a decade of increasing sophistication in the field of recombinant DNA technology, application of recombinant techniques to modification of the genetic structure of higher plants appears to be the most resistant to predictability and even threshold accomplishment of desired results. It has been possible for some time to transform prokaryotes and to convey to them desirable properties relating to manufacture of foreign proteins; more recently, eukaryotic microorganisms, such as yeast, have been successfully transformed to adapt them to desirable characteristics, and, perhaps most recently, vertebrate cells in tissue culture have been employed as host cells for transformation with recombinant expression vectors. However, although there have been some successes, techniques for succ ssful transformation of plant cells

have remained elusive. It has not even been possible to maintain plant-derived cell lines in "tissue culture" conditions and then regenerate intact plants except for a few species of plants, notably tobacco, petunias, and cotton.

Successful, reproducible transformation of plant cells, combined with subsequent regeneration of the plant tissue into "normal" plants to allow these to function according to the normal agrarian reproductive cycle carries the possibility of some highly desirable results. For example, plants transformed with genes which, when expressed, would confer resistance upon the plant to predation by insects, to weed killers, or to infection by plant diseases would result in a far more efficient agricultural and forestation system. Accordingly, straightforward ways to assure the incorporation of foreign genetic material into plant tissues on a permanent basis have long been sought.

Possibly because of the rigidity and limited permeability of plant cell walls, direct transformation of plant cells with "naked" DNA has proved impossible. Even after removal of the cell walls, and culturing of the resultant protoplasts, it appears that plant cells are refractory to integration of foreign naked DNA. Thus, efforts have focused on co-opting methods for DNA insertion which already exist in nature. Notable among these is the capacity of Agrobacterium tumefaciens to infect dicotyledonous plants and to confer on them genetic alterations which result in the growth of what is commonly known as crown gall tumor.

Some factors associated with the integration of Agrobacterium DNA into infected plants are now understood. First, it is clear that the bacterial DNA

transforming the infected plant cell is non-chromosomal DNA; and is transferred from a plasmid vector (Ti).

At least two Ti (tumor inducing) plasmid types (which are designated according to the opines associated with them) have been mapped and studied extensively: "nopaline" Ti plasmids such as pTiT37 (Depicker, A., et al, J Molec Appl Genet (1982) 1:561; Bevan, M., et al, Nucleic Acids Res (1983) 11:369) and pTiC58 (see, e.g., Shaw, C. H., et al, Gene (1983) 23:315); and "octopine" plasmids such as pTiB6S3 (DeGreve, H., et al, J Molec Appl Genet (1982) 1:499). They are quite large (e.g., 132 Mdal or about 200 kilobases (kb) for pTiC58) and have a number of relevant features. There is, in each case, a "virulence" (vir) region which appears to be associated with those functions permitting successful infection and integration of certain DNA portions into the host plant cell, i.e., this region is associated with encoding whatever products effect the attachment of the bacterium to a plant cell, transfer of the plasmid DNA into the plant cell, or other necessary functions required for infection. A second region of significance is the "transfer-DNA" or T-DNA region constituting that portion of the plasmid which, after infection, actually becomes integrated into the plant genome.

In a wild type Ti plasmid, this T-region has a number of important characteristics. First, it generally contains an opine synthase region which, when expressed, provides the enzymes required for the synthesis of a characteristic opine--nopaline in the case of the nopaline-type plasmids, and octopine in the case of octopine-type plasmids. (Nopaline is a conjugate of arginine and alpha ketoglutaric acid; octopine is a conjugate of arginine and pyruvic acid.) Presumably, synthesis of these opines serves a natural function in

the normal course of infecti n--an additional region of the plasmid encodes enzymes which permit the degradation and metabolism of the particular opine encoded by the synthase in the T-DNA region. Thus, plant cells infected by a particular plasmid acquire the ability to synthesize a particular opine, i.e., nopaline or octopine, which product then is a nutrient for additional infecting cells. That is, the ability to metabolize a particular opine is encoded on the same plasmid as the synthase encoding region, but not integrated into the host cells' genome.

The T-DNA region also encodes genes which appear to affect endogenous levels of the plant hormones cytokinin and auxin. This is doubly significant. On the positive side, this function is useful in selecting successful transformants in tissue culture, since generally only transformed cells have the capacity to synthesize these hormones and are capable of reproduction in their absence from the medium. On the other hand, the presence of these specific genes in the transferred DNA has natural consequences in the course of infection, and because of the increased and unbalanced amounts of plant hormones, transformed plant cells fail to regenerate into complete normal plants and infected intact plants develop crown galls. Indeed, it has been shown that if specific mutations are made in the T-DNA regions affecting the levels of these hormones, sufficient imbalance of the auxin-cytokinin ratio can be obtained to result in growth predominantly of roots or predominantly of shoots from the resulting tumor tissue.

Finally, the T-DNA is flanked by "border" sequences which contain approximately 25 nucleotide direct repeats. Because of the nature of these sequences, it is surmised, although not known, that these

regions may be involved in the insertion of the intervening T-DNA into the chromosome of the infected cell.

Because the T-DNA region is integrated into the host cell chromosome, attempts have been made to utilize this region as a vehicle for transformation of plant host cells by foreign DNA. None of these attempts have been entirely successful, if evaluated for the goal of obtaining the desired combination of an expressed foreign gene in the context of a normal plant.

Shaw, C. H., et al, Gene (1983) 23:315 succeeded in transforming an axenic culture of tobacco seedlings by infecting them with A. tumefaciens presumably containing a recombined Ti plasmid. This plasmid was thought to result from an in vivo cross-over in the A. tumefaciens cells involving homologous regions of the T-DNA. The putative recombined vector was derived from a broad-host range intermediate vector containing the HindIII-23 right-end T region fragment of the nopaline plasmid pTiC58 into which the rabbit β-globin gene had been inserted, and the Ti plasmid (also pTiC58) indigenous to the Agrobacterium. The infected seedlings incorporated the β-globin DNA, but did not make any transcripts of it.

Herrera-Estrella, L., et al, Nature (1983) 303:209 succeeded in obtaining expression of the coding sequence for a heterologous protein conferring chloramphenicol resistance in axenic cultures derived from Agrobacterium infection induced-tobacco seedling tumors. The coding sequence for chloramphenicol acetyl transferase (CAT) was linked to the promoter sequences of the nopaline synthase gene to obtain a NOS-CAT chimaeric gene and placed in a recombinant vector with a narrow host range (E. coli) origin of replication, a

region of T-DNA homology, and the ampicillin resistance conferring (Amp^R) sequences. This plasmid was transformed into A. tumefaciens harboring pTiC58 and successful transformants selected for Amp^R, thus insuring that recombination between the bacterial vector and Ti plasmid had occurred. When the Agrobacterium containing the presumed recombined plasmid was used to infect tobacco seedlings, the resulting tumor cells, grown in tissue culture were not regenerated into plants, but were able to produce the CAT-encoded protein.

Fraley, R. T., et al, Proc Natl Acad Sci (USA) (1983) 80:4803 disclose transformation of petunia protoplasts by co-cultivation with A. tumefaciens selected, analogously to the work of the preceding paragraph, for an in vivo recombination of two vectors: the first a narrow host range plasmid vector containing a chimaeric gene having a coding sequence for a protein conferring neomycin resistance linked to the nopaline synthase promoter, and having a sequence homologous to pTiB6S3 to encourage recombination, and the second indigenous Ti plasmid pTiB6S3. Protoplasts were obtained which were resistant to the aminoglycoside antibiotic, kanamycin, normally toxic to plant cells. It was assumed that resistance is conferred by the inserted gene. Normal plants were not regenerated.

It has also been shown by de Framond, A. J., Biotechnology (1983) 262-269, and by Hoekema, A., et al, Nature (1983) 303:179 that the virulence function and T-DNA functions of the Ti plasmid can be separated into a bipartite system which will cotransform plant cells upon infection and successfully produce tumors.

Finally, Barton, K. A., et al, Cell (1983) 32:1033 discloses experiments which show that the DNA sequence of alcohol dehydrogenase (ADH) from yeast

inserted into the "rooty" locus of a Ti plasmid is found in multiple copies in DNA isolated from plants regenerated after infection, although the cells are not tumorous--i.e., they required added cytokinin for growth. Seeds obtained from self pollination of these plants also generated normal tobacco plants, except, apparently, for the inclusion of approximately 20 copies of the ADH coding sequence in their DNA. The ADH was not expressed. As the plants remain normal after the transformation with respect to tumor formation, the vector used for transformation was referred to as "disarmed".

In none of the foregoing disclosures has it been possible to show both expression and regeneration of normal plants in the same experiment. The present invention provides a means to obtain this desirable result. It also offers greater control over the nature of the expression obtained by permitting standard recombinant DNA techniques to be employed in constructing successful transformation vectors without the necessity for selecting for an in vivo recombination event.

Disclosure of the Invention

The present invention in one aspect relates to vectors useful for expression of foreign gene sequences in plant tissues. A basic intermediate carrier vector behaves as a receiving unit which permits the introduction of any desired foreign gene sequence so as to be suitably disposed to take advantage of promoter and polyadenylation signal sequences which are operable in plant tissues. A wide variety of foreign genes can be introduced with facility. Further, when such desired gene sequences have been inserted, the resulting expression carrier vectors can be spliced using standard recombinant DNA restriction and ligation techniques to contain any

desired number of expression cassettes for a variety of genes. Among these cassettes, it may be desirable to include a marker which will permit easy selection of successfully transformed cells.

The foregoing expression carrier vectors, when transformed directly into plant tissue, unlike the recombined vectors employed in the standard Ti vector transformation techniques, do not produce tumors, and plant cells transformed with these carrier vectors alone are capable both of normal regeneration and of expression of the desired gene.

Accordingly, in one aspect, the present invention relates to expression carrier vectors effective in expressing the coding sequence of foreign genes in recombinant plant cells and to the intermediate DNA sequences and vectors useful in constructing them. Such expression carrier vectors include:

1. Recombinant vectors containing one or more expression cassettes, each such expression cassette comprising a promoter normally operable in plant cells and a polyadenylation signal operable in plant cells, both operably linked to a coding sequence for a desired foreign protein. The expression carrier vectors may contain more than one such expression cassette. These vectors also contain at least one cassette-unique restriction site at the terminus of at least one expression cassette. This class of vectors is useful in obtaining the expression of the foreign coding sequences in plant cells after direct transformation of such cells with these vectors.

2. Another class of expression carrier vectors comprises those of the preceding paragraph, but modified so as to contain at least one A. tumefaciens T-DNA border sequence proximate a terminus of the expression cassette,

or if multiple expression cassettes are contained in the vector, at a terminus of these series. Preferably, the single expression cassette, or the series of expression cassettes, is framed by two such T-DNA border sequences. These vectors also contain, on their vector portions, a broad host range bacterial origin of replication. They are useful in transforming plant cells so as to obtain expression of foreign protein, either by the direct transformation methods employed in connection with the vectors of the previous paragraph, or in transformation methods which involve the mediation of the virulence regions of an Agrobacterium Ti plasmid.

In other aspects, the invention relates to intermediate plasmids and DNA sequences employed in the construction of the foregoing vectors, to methods of transforming plants or plant cells using these vectors, to plants or plant cells transformed with such vectors, to methods of producing foreign proteins using plants so transformed, and to the proteins so produced.

Brief Description of the Drawings

Figure 1 shows the construction of pCMC59 and pCMC60.

Figure 2 shows the construction of pCMC121.

Figure 3 shows the construction of pCMC101.

Figure 4 shows the construction of pCMC91.

Figure 5 shows the construction of pCMC72.

Modes of Carrying Out the Invention

A. Definitions

As used herein, a promoter sequence "normally operable in plant cells" refers to a promoter sequence which is not only coincidentally compatible with plant cells in the sense of being minimally capable of

initiating transcription of a coding sequence into mRNA, but which is found natively to effect gene expression in plant tissue. Such promoter may be of plant origin; however, more typically in the embodiments of the invention it is, in fact, a bacterial sequence. Ordinarily, the sequence may be relatively silent in the bacterium and is designed to operate specifically in an infected plant cell. For example, many of the vectors of the present invention utilize the promoter for nopaline synthase, which is carried on an Agrobacterium tumefaciens plasmid, but which has not been reported to express in the bacterium. However, when the plasmid is introduced into infected plant cells, it operates in this context to express the nopaline synthase coding sequence. These promoters are, although not of plant origin, "normally operable" therein.

"Polyadenylation signal operable in plant cells" refers to a sequence normally found 3' of a coding sequence which appears to be necessary for its transcription, transport of the polyA-mRNA from the nucleus and its stable accumulation in the cytoplasm, permitting subsequent translation in eukaryotic cells. It is currently believed that the function of these sequences is to signal polyadenylation of the messenger RNA transcribed from the coding sequence. However, the complete function of these signal sequences has not been unequivocally established. Therefore, as used herein, this term refers to those signals which follow the coding sequence in DNA and assure its expression, including translation, in the eukaryotic cellular environment. It is not at present clear whether this ability to assure expression in plants is confined to sequences which are found to be so utilized by plant cells in nature. Therefore, any sequence which in fact is successful in

performing this function in plant cells is includ d in the definition.

"Polylinker" refers to a DNA sequence which contains at least three closely associated restriction enzyme sites after ligation into a receptor DNA sequence.

"Expression cassette" refers to a DNA sequence which is capable of effecting expression of a contained coding sequence in plants. Thus, a typical expression cassette contains a promoter sequence normally operable in plant cells, operably linked to the coding sequence and a polyadenylation signal operable in plant cells also operably linked to the coding sequence. The word "cassette" is also used in connection with other DNA sequences which are designed to be treated as a unit. For example, a series of "expression cassettes" would also be referred to as a "DNA sequence cassette".

"Cassette-unique" restriction site refers to an endonuclease enzyme cleavage recognition site which is found only once in the referenced cassette or series of cassettes.

"Vector sequence" or "vector segment" or "vector fragment" refers to DNA sequences on a plasmid that are not part of an expression cassette, DNA sequence cassette, or other "cassette" DNA sequence. Typical of "vector sequences" are origins of replication and T-DNA border sequences.

"Narrow host range bacterial origin of replication" refers to an origin of replication which is capable of function only in a very limited number of bacterial species. Typically, in most recombinant work, such replicons are confined to functionality in such commonly used species as E. coli, and, in the context of the present invention, fail to replicate in foreign hosts such as Agrobacterium. Conversely, "broad host range

bacterial origin f replication" refers to those replicons which are functional not only in their strain of origin but also in disparate bacterial species. In the context of the present invention, the most meaningful example would be those origins which are capable of replication both in E. coli and in Agrobacterium.

As used herein, "T-DNA border sequences" refers to repeating DNA sequences the same as, or analogous to, those bordering the T-DNA portion of the Ti plasmids of A. tumefaciens. In the wild type A. tumefaciens, these repeating sequences frame the T-DNA which is transferred into the chromosome of the infected cell. The definition of border sequences as used in this invention is not limited to these specific repeating segments, but includes any modifications thereof which still are functional in mediating the transfer of the intervening material, with the aid of the virulence region of a Ti plasmid, into a host cell genome. The abbreviations "LB" (left border) and "RB" (right border) are used to designate those sequences framing the T-DNA of the A. tumefaciens plasmid.

"Plants" or "plant cells" refer to plant types which are capable of reproduction by formation of seeds, and these terms include the plant itself, the cells of the plant, protoplasts which derived from plant cells, other plant-derived cells such as tissue culture cells, tumor cells, calli and seeds, and progeny thereof. It is understood that progeny can undergo spontaneous or intentional modifications when cultured over several generations, and such modified progeny are intended to be included as long as they are derived from the plant cell or plant referred to.

"Operably linked" refers to sequences which are juxtaposed in such a way that their functionality is

maintained. Thus, a promoter "operably linked" to a coding sequences refers to a disposition of a promoter which is capable of effecting the transcription and translation of this sequence. Similarly, a polyadenylation signal "operably linked" to a coding sequence refers to a disposition of this signal which permits production of protein from the coding sequence.

B. General Description and Preferred Embodiments

The transformation techniques of the present invention utilize a system of vector constructs which permits facile manipulation of gene sequences in an interchanageable set of vectors of general utility.

B.1 The Intermediate Carrier Vector

The seminal vector is an intermediate carrier vector which is designed to permit convenient insertion of any desired polypeptide encoding sequence.

This vector comprises a "cassette" DNA sequence which includes, in order, proceeding 5'-3' in the sense strand:

(a) a first cassette-unique restriction site;

(b) a promoter sequence (oriented 5'-3') which is normally operable in plant cells;

(c) a polylinker;

(d) a polyadenylation signal (oriented 5'-3') operable in plant cells; and

(e) a second cassette-unique restriction site.

The presence of the polylinker permits easy manipulation in relation to coding sequences so as to permit insertion either of an ATG-initiated sequence encoding a "mature" protein or of a sequence into reading frame with a partial coding sequence operably included with the promoter.

Upon insertion of such coding sequences this vector becomes an expression carrier vector capable of expressing the coding sequence when transformed into plant cells. Carrier vectors containing a wide variety of coding sequences, for example, APH-I and APH-II (which encode proteins conferring resistance to the aminoglycoside antibiotic G418 or kanamycin, which are toxic to yeast, plant and mammalian as well as to bacterial cells); beta interferon, which may, in fact, protect plants as well as mammals against viral infection (Orchansky, P. et al, Proc Natl Acad Sci (USA) (1982) 79:2278; Bt-toxin which acts as an internal pesticide for plant tissues; and CAT which catalyzes an acetylation modification of chloramphenicol. This list is far from exhaustive, and any coding sequence which can be provided with its own ATG start codon and framed by suitable restriction sites can be utilized. In addition, where the intermediate vector which contains the promoter sequence also contains, operably linked to the promoter, codons which include an ATG start signal, any sequence which can be placed in reading frame with the ATG can be expessed as a fusion protein. For example, a portion of the β-galactosidase coding sequence has been so ligated. Thus, a panoply of carrier expression vectors for many desired gene sequences can be constructed from an intermediate carrier vector.

B.2 Expression Carrier Vectors for Direct Transformation

The initial single cassette carrier expression vectors which result from insertion of coding sequences into the intermediate carrier vector are further characterized in that the expression cassettes contained in them are interchangeable and c mbinations of these can

be used to form carrier expression vectors which have two, three or any desired number of cassettes. Thus, it is possible to construct, for example, in a straightforward fashion, a vector which contains not only a cassette for a desired gene sequence, but also a cassette for the expression of a selectable marker which permits selection of the successfully transformed cells.

The critical portions of the expression carrier vector comprise one or more expression cassettes, each of which contains a desired coding sequence operably linked both to a promoter normally operable in plant cells, and to a polyadenylation signal operable in plant cells. In a preferred embodiment, at least one of the desired coding sequences encodes a protein which results in a phenotypic characteristic enabling selection of transformed plant cells--i.e., a dominant selectable marker. Also preferred are those embodiments wherein a cassette-unique restriction site is located at one end of the expression cassette in a single expression cassette vector, and at the terminus of a series of expression cassettes or at the junction between two cassettes in a multiple cassette vector, and is, in this case, unique not only to the particular expression cassette of its location, but to the entire series or "DNA sequence cassette". In instances where the expression carrier vector is to be used as a donor vector for supplying an additional expression cassette to a recipient, it is further preferred that there be a second cassette-unique restriction site at the expression cassette's other terminus.

In a preferred method of construction of expression carrier vectors which contain more than one expression cassette, two single expression cassette vectors are used, one to provide simply an expression

cassette (a donor) and the other t pr vide its own expression cassette as well as the vector sequences (the recipient). In this method, the recipient vector is cleaved with one restriction enzyme which cuts at a cassette-unique site at the terminus of its expression cassette; the donor expression cassette is excised by treating the donor plasmid with two restriction enzymes, one for each cassette-unique restriction site at the ends of the expression cassette. The excised expression cassette and the recipient plasmid are then ligated using standard conditions to yield the resulting multiple expression cassette vector.

If the cassette-unique restriction sites are chosen so that they yield compatible sticky ends (for example, XhoI and SalI), the inserted expression cassette can be oriented in either of two orientations, and the position of the regenerated cassette-unique restriction site will depend on which orientation has resulted. For example, in the examples below, the expression cassettes are bounded by XhoI and SalI restriction sites at the 5' and the 3' termini, respectively. The recipient vector is treated with SalI to form linear DNA with SalI sticky ends. The excised donor expression cassette bears XhoI and SalI sticky ends. Under these circumstances, if the expression cassette is oriented so that the DNA sequence is in the same direction as the recipient, a SalI site will be regenerated at the terminus of the resulting DNA sequence double cassette; if it is oriented in the opposite direction, the cassette-unique SalI site will be located between the two expression cassettes. Both orientations will be functional, and both will provide a suitable cassette-unique restriction site to permit the double expression cassette vector to serve as a recipient for the insertion of additional expression cassettes.

In summary, an intermediate carrier vector is constructed and is part of the invention. It has, reading in the 5' to 3' direction of the sense strand, a first restriction site, a promoter normally operable in plants, a polylinker, a polyadenylation signal operable in plants, and a second restriction site. The polylinker permits convenient insertion of a desired coding sequence to generate a single cassette expression carrier vector wherein the single cassette is bounded by the two restriction sites. Mutiple cassette expression carrier vectors can be constructed by excising the expression cassette of one such vector which has been cleaved at these two restriction sites and inserting it into another using just one of the restriction sites of the recipient vector, a procedure which can be repeated any desired number of times. In a particularly preferred embodiment, this process is made more efficient by utilizing combinations of restriction sites such that one of the sites is not regenerated after the ligation of two expression cassettes.

The nature of remaining vector fragment portions of the expression carrier vectors just described, which are suitable for direct transformation into plant tissues under appropriate conditions, is less critical than that required for other transformation techniques, i.e., they need not contain sequences which would be required for transformation using the mediation of an infecting bacterium. Thus, these vectors may or may not contain T-DNA border sequences from A. tumefaciens, and their origins of replication may be adaptable to either a narrow or broad range of hosts. In other words, the Agrobacterium-dependent carrier expression vectors described in paragraph B.3 below can be used as "ordinary" expression carrier v ctors in

direct transformation. All of the illustrative paragraphs below describe expression carrier vectors which are suitable for direct transformation. Examples of vectors constructed in paragraph G. describe such vectors which are appropriate for both direct transformation and for Agrobacterium-mediated transformation.

B.3 Expression Carrier Vectors Useful in Agrobacterium-Mediated Transformation

The expression carrier vectors described above can be modified in their vector segments so as to adapt them for use in plant cell transformation mediated by bacteria. If A. tumefaciens is to be used as the infectious agent, the modified vector will have at least one A. tumefaciens T-DNA border sequence disposed proximate the terminus of the expression cassette or the series thereof. Preferably the expression cassette or series is framed by a pair of such border sequences. In order to permit the vector to replicate in the A. tumefaciens host, while still permitting the vector to have been constructed in, for example, E. coli, the vector segment contains a broad host range bacterial origin of replication. While these modifications do not impair the ability of the expression carrier vectors to transform plant cells directly, they are necessary to permit transformation using the offices of the intermediate bacterium.

In construction of the modified vectors, the preferred pivotal vector is comprised of an expression cassette containing a desired coding sequence (preferably that encoding a dominant selectable marker) operably linked both to a promoter normally operable in plant cells and a polyadenylation sequence operable in plant

cells framed by the right border (RB) and left border (LB) sequences associated with A. tumefaciens. A cassette-unique restriction site is disposed between one of the border sequences and the terminus of the expression cassette. The vector segment contains a broad host range bacterial origin of replication.

This basic expression carrier Agrobacterium-adapted vector is exemplified below by pCMC91. In pCMC91, the coding sequence encodes the dominant selectable marker, a modifed APH-II, conferring resistance against G418 and kanamycin. Derivatives of the basic construction contain additional expression cassettes so that these derivatives are useful in expressing the coding sequence of any desired foreign gene in the transformed plant cell, and, further, still retain the selectable marker feature of the original cassette. These expression cassettes can be conveniently derived from the vectors already described above in paragraph B.2. They are excised by use of the two cassette-unique restriction sites at either end of the cassette, and ligation of the cassette at the cassette-unique restriction site between the border sequence and the terminus of the APH-II cassette in the recipient vector.

B.4 Methods of Transforming Plant Cells Using Vectors of the Invention

B.4.a. Direct Transformation

All of the expression carrier vectors of the invention can be used to transform plant cells directly. In this method, no bacterial infection is employed. Rather, plant cells are treated under suitable conditions directly with naked DNA, i.e., simply with a

suspension of the appropriate vector. At present, one workable method to effect this transformation is to suspend plant cell protoplasts with the plasmid DNA in the presence of a suitable facilitator such as, for example, polyethylene glycol (PEG). This technique is described in greater detail in paragraph H. below. Another method, which has been used less frequently, employs microinjection into individual plant cells. This method is more tedious, but does not require the preparation of protoplasts. In any event, the vectors of the invention are suitable for any workable direct transformation technique and it should be understood that the invention is not limited to their use in the foregoing specific methods.

B.4.b. Bacterial Mediated Transformation

Those vectors which are described in paragraph B.3 are suitable for use in bacterial mediated transformation of plant cell hosts. Two variations of such methods are commonly employed. Both share the same initial step--transformation of a suitable bacterial host harboring a Ti plasmid bearing the virulence coding regions. The bacterial host, now containing both the expression carrier vector and the virulence-containing plasmid, is either co-cultivated with plant cells, preferably protoplasts, in tissue culture, or injected directly into wounded plant tissue. These alternatives are described in greater detail below.

Whichever alternative is used, the infecting or co-cultivated Agrobacterium will contain both the expression carrier vector and a plasmid bearing the virulence region normally associated with a tumor-inducing plasmid. It is preferred, in order to prevent

the resulting transformed cells from becoming tumorous, that the virulence region reside on a plasmid lacking the tumor-inducing functions normally carried in its T-DNA region. Such a disarmed virulence region containing plasmid may already be present in the host bacterial cell as in the example hereinbelow, or, perhaps less conveniently, a bacterial host devoid of tumor-inducing plasmids may be transformed both with the carrier expression vector and a disarmed virulence-encoding vector, either simultaneously or sequentially. In a preferred embodiment, an A. tumefaciens host already containing a disarmed Ti plasmid is used as the recipient for the expression carrier vector.

To summarize, four illustrative methods of transformation are:

(1) The carrier expression vector is suspended under suitable conditions with plant cell protoplasts to permit direct transformation.

(2) Microinjection.

If methods (1) or (2) are used, it is immaterial whether the replicon is of narrow or broad host range, and whether border sequences are present. The vectors of both B.2 and B.3 may be employed.

(3) The carrier expression vector is first transformed or conjugated into Agrobacterium containing a disarmed Ti vector and then the Agrobacterium co-cultivated with plant protoplasts in tissue culture.

(4) The carrier expression vector is first transformed into Agrobacterium as above and the Agrobacterium applied directly into wounded plant tissue.

Methods (3) and (4) are suitable only for the vectors of B.3.

C. Methods Employed for Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g, New England Biolabs, Product Catalog. In general, about 1 µg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 µl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Partial digestions are occasionally specified. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform; this step may be followed by ether extraction and the nucleic acid recovered from aqueous fractions by precipitation with ethanol followed by running over a Sephadex G-50 spin column. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four

nucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 0.1 mM dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back single strands, even though the four dNTPs are present, at 3' sticky ends. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated followed by running over a Sephadex G-50 spin column. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

Synthetic oligonucleotides are prepared by the triester method of Matteucci, et al; (J Am Chem Soc (1981) 103:3185-3191). Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles γ^{32} P ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Ligations are performed in 15-30 l volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 µg/ml BSA, 10 mM-50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 µg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar

excess of linkers) are performed at 1 μM total ends concentration.

In vector construction employing "vector fragments," the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{+2} using about 1 unit of BAP per μg of vector at 60° for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated and desalted by application to a Sephadex G-50 spin column. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction of the unwanted fragments.

In the constructions set forth below, correct ligations for plasmid construction are confirmed by transforming E. coli strain MM294 (Talmadge, K., et al, Gene (1980) 12:235; Meselson, M., et al, Nature (1968) 217:1110) obtained from E. coli Genetic Stock Center, CGSC #6135, or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D. B., et al, Proc Natl Acad Sci (1969) 62:1159, following chloramphenicol amplification when feasible (Clewell, D. B., J Bacteriol (1972) 110:667) and analyzed by restriction and/or sequenced by the dideoxy method of Sanger, F., et al, Proc Natl Acad Sci (USA) (1977) 74:5463, as further described in Messing, et al, Nucleic Acids Res (1981) 9:309, or by the

method of Maxam, et al, Methods in Enzymology (1980) 65:499.

The following examples illustrate the construction of suitable intermediate carrier vectors, single cassette and double cassette expression carrier vectors, and bacterial adapted derivatives thereof suitable for the transformation techniques described. In these vectors, the nopaline synthase (NOS) promoter of pTiT37 is utilized along with the polyadenylation signal for this gene. Of course, other promoter sequences normally operable in plant cells can be used as well as other polyadenylation signals operable in plants. Suitable alternate promoters include the maize promoter for alcohol dehydrogenase-1 or alchohol dehydrogenase-2 (Gerlach, W. L., et al, Proc Natl Acad Sci (USA) (1982) 79:2981), cauliflower mosaic virus promoters (Daubert, S., et al Virology (1982) 122:444), and wheat promoter associated with the small subunit of ribulose biphosphate carboxylase (Broglie, R., et al, Biotechnology (1983) 1:55). The foregoing are readily available in the art, but, of course, other suitable promoters can be used. Other polyadenylation signals which are available presently include those found on any of the above genes, or those of Schuler, et al (Schuler, M. S., et al, Nucleic Acid Res (1982) 10:8225). The coding sequences inserted below also are not limiting. Any available coding sequence could be substituted, either with an ATG start codon so as to result in an mature protein, or in reading frame with a portion of the NOS sequence.

D. Construction of the Intermediate Carrier Vectors pCMC60 (and pCMC59)

The construction of the intermediate carrier vector pCMC60 is outlined in Figure 1. This plasmid is

constructed by manipulating the nopaline synthase (NOS) gene derived from the 3.2 kilobase (kb) HindIII-23 DNA fragment in the T-DNA region of A. tumefaciens Ti plasmid pTiT37 (Bevan, M., et al, Nucleic Acid Res (1983) 11:369; Depicker, A., et al, J Mol Appl Genet (1982) 1:5613). The HindIII-23 fragment, containing the complete NOS gene, obtained from pTiT37, was cloned into the HindIII site of pBR325 (Bolivar, F., et al, Gene (1978) 4:121) to obtain the starting plasmid pCMC1 (8.9 kb). pCMC1 served as the source of the NOS promoter, polyadenylation signal, and in the bacterial adapted plasmid derivatives, the "right" T-DNA border sequences.

The construction of pCMC60 employs a number of intermediate plasmids as shown in Figure 1 and below. These are: pCMC30 which provides the NOS promoter modified to contain a HindIII site at its 3' terminus, pCMC39 which provides the NOS promoter modified to contain a XhoI site at its 5' terminus, and pCMC49 which provides the polylinker, the polyadenylation signal and a bacterial host replicon.

D.1 pCMC30

pCMC30 was constructed to contain the NOS promoter modified to contain a HindIII site at the 3' terminus. A primer repair reaction, essentially as outlined by Messing, J., Third Cleveland Symposium on Macromolecules: Recombinant DNA (1981). A. Wiseton, ed., Elsevier Amsterdam, pp.143-153; was employed to effect this modification. This was done as follows: pCMC1 was digested with Sau3AI to excise a 346 bp fragment containing the NOS promoter. This fragment was isolated and cloned into the BamHI site of the single stranded M13 bacteriophage mp10RF (Messing, J., et al, Gene (1982) 19:269) to produce mCMC10, a 7.57 kb bacteriophage,

whose construction was confirmed by digestion with SstII and HindIII to release the expected 260 bp fragment.

The sequence of the Sau3AI insert was confirmed to comprise the NOS promoter and the sequences surrounding the 3' terminus of the promoter were confirmed by dideoxy sequencing to conform to the expected sequence as disclosed by Depicker, A., et al, J Mol Appl Genet (1982), Vol. 1, p. 566. The 3' terminus of the promoter was modified using a primer repair reaction by annealing with the synthetic pentadecamer 5'-pTTGCAGATTATTTGG-OH 3' which complements this fragment so as to include the first A of the ATG codon and 14 upstream nucleotides. In the primer repair, 50 µg of single-stranded mCMC10 DNA was digested with HaeIII and the 597 bp fragment isolated. One µg of the isolated fragment was annealed with the pentadecamer (supra) and extended with T4 DNA polymerase I in the presence of dNTPs under standard conditions. The remaining single-stranded region at the 3' end was simultaneously degraded to the 5' terminus of the annealed primer. The resulting primer-repaired double-stranded fragment thus terminates at the 3' end after the A of the ATG codon at position 1 of the NOS coding sequence. This double-stranded fragment was digested with EcoRI and the 320 bp EcoRI/blunt DNA fragment purified and cloned into pUC13 (a freely available pBR322 derivative containing polylinkers; see Messing, J., et al, Gene (1982) 19:269) as follows:

pUC13 (10 µg) was digested with HindIII and then treated with DNA polymerase I (Klenow fragment) in the presence of dNTPs and then digested with EcoRI. The vector fragment was ligated with the 320 bp EcoRI/blunt promoter fragment above and the ligation mixture transformed into E. coli MM294. Successful transformants

were selected by Amp^R, and the required plasmid, pCMC30, confirmed by restriction analysis with SstII/HindIII to yield the expected 188 bp DNA fragment.

The correct construction was further confirmed by dideoxy sequencing to contain a recreated HindIII site at the junction of the insert with pUC13; the plasmid also provides EcoRI, SstI, and SmaI/XmaI sites 5' to the promoter sequences. Thus, pCMC30 provides the NOS promoter as a convenient EcoRI, SstI, or SmaI (XmaI)/ HindIII cassette.

D.2 pCMC39

pCMC39 is another intermediate plasmid and it contains the NOS promoter modified at the 5' terminus to contain a XhoI site. The XhoI site was excised from pBW14 (4375 bp), which is a pBR322 derivative into which a XhoI-containing octanucleotide (5'-CCTCGAGG-3') was ligated into a Klenow repaired, HindIII digest of pBR322. pBW14 was digested with XhoI, treated with DNA polymerase I (Klenow fragment) in the presence of dNTPs and then treated with EcoRI. The approximately 40 bp EcoRI/XhoI (blunt) fragment was isolated, and ligated in a two-stage ligation to a SmaI/EcoRI digest of pCMC29. pCMC29 contains an unmodified NOS promoter whih was originally obtained from pCMC1 by digestion of pCMC1 with Sau3AI and inserted into the BamHI site of pUC13. The ligation mixture was used to transform *E. coli* MM294 and Amp^R colonies were selected. Successful transformants were screened for the desired 3.1 kb plasmid, designated pCMC39.

pCMC39, therefore, contains the NOS promoter modified at the 5' terminus to contain a XhoI site immediately upstream as shown in Figure 1. Its construction was confirmed by restriction analysis.

D.3 pCMC49

pCMC49 was constructed to contain the NOS polyadenylation signal and a polylinker segment through the intermediate plasmid pCMC20, which, in turn, was constructed using a digest of pCMC1 as the source of the relevant sequences, combined with rsal03 used as a source of the polylinker for the expression plasmid. pCMC1 was digested with Sau3AI and the 250 bp fragment containing the polyadenylation signal from the NOS gene isolated. The isolated fragment was ligated with BamHI digested pUC8 (a freely available pBR322 derivative containing a polylinker for convenient constructions; see Messing, J., et al (supra)) and the ligation mixture was transformed into E. coli MM294. Successful (Amp^R) transformants were screened for the expected 3.0 kb plasmid and construction was confirmed by restriction analysis. pCMC20 contains unique SstI and SmaI (XmaI) sites 5' to the polyadenylation signal and SalI, PstI, HaeII, HindIII and BssHII sites 3' to the signal.

To give pCMC49, pCMC20 was digested with XmaI, blunt-ended with Klenow fragment and the four dNTPs, and then digested with SalI. The 260 bp desired terminator signal fragment was purified and ligated to BamHI(Klenow repaired)/SalI digested rsal03 purified vector-plus-polylinker fragment and the ligation mixture transformed into E. coli MM294. Amp^R transformants were selected and screened for the desired plasmid, pCMC49, which contains the signal fragment framed by a 5' polylinker containing HindIII, XbaI, BglII, PstI, BamHI and XmaI/(SmaI) sites, and a 3' SalI site as well as the origin of replication from rsal03. (rsal03 is a pBR322 derivative in which (i) a polylinker containing HindIII XbaI, BglII, PstI, BamHI, and SalI sites is substituted for the 621 bp HindIII/SalI vector fragment in the tetracycline resistance region, by

insertion of the small HindIII-BamHI fragment from VX (Seed, B., Nucleic Acid Res (1983) 11:2427) into the large HindIII-BamHI fragment of pBR322, and (ii) the 514 bp RsaI DNA fragment from bacteriophage M13RF containing the origin of replication is adapted with HindIII linkers, cut with HindIII and inserted at the HindIII site. In rsal03, the M13RF origin of replication is oriented in the same direction as the bla gene on the pBR322 portion.)

D.4 Completion of pCMC60

The 3' unmodified, XhoI-5' supplemented, NOS promoter from pCMC39 and the polylinker-signal sequences along with the rsal03 vector fragment from pCMC49 were ligated to give pCMC59, the penultimate plasmid in the preparation of pCMC60, which is also a (modified) intermediate carrier vector. To do this, pCMC39 was digested with EcoRI and HindIII, the digest ligated to EcoRI/HindIII digested pCMC49 and the ligation mixture used to transform E. coli MM294. Amp^R colonies were screened for the presence of a 4.48 kb plasmid containing unique EcoRI and HindIII sites as well as the remaining expected restriction sites.

The selected plasmid, pCMC59, contains the 3' unmodifed NOS promoter preceded by several 5' restriction sites which is upstream from the polylinker and the polyadenylation signals followed at the 3' terminus by additional restriction sites as well as the vector fragment of rsal03, which includes the Amp^R gene and replicon. pCMC59 is an alternate form of intermediate carrier vector which permits the insertion of a desired coding sequence so as to yield a fusion protein with the first approximately 15 amino acids of

the NOS sequence provided the sequence is inserted in appropriate reading frame.

The more convenient intermediate carrier vector, pCMC60, which permits the insertion of a coding sequence for a mature protein in front of the HindIII site provided by the modified promoter was constructed employing pCMC59 and pCMC30. pCMC30 containing the 3'-modified NOS promoter was digested with SstII and HindIII and the 188 bp promoter-containing fragment isolated. pCMC59 was also digested with SstII and HindIII and the vector fragment purified. A ligation mixture of these two purified fragments was transformed into E. coli MM294 and selected on medium, containing 100 µg per ml ampicillin and 40 µg per ml X-gal, for white, Amp^R colonies. (Colonies which are Amp^R due to undesired pCMC30 transformation are blue; pCMC30 contains the lac operator which results in depression of β-galactosidase in MM294, thus giving blue colonies on X-gal plates, if present). White Amp^R colonies were screened for transformants containing the desired construction, pCMC60.

Thus, pCMC60 contains a narrow host range bacterial host vector segment from rsal03 and a gene control sequence cassette which, in a 5' to 3' direction, contains a cassette-unique restriction site (a XhoI site, among others), the NOS promoter modified at its 3' end to contain a HindIII site immediately preceding what would be the ATG initiation codon in the Ti plasmid nopaline synthase gene, a polylinker segment, and a polyadenylation signal derived from the NOS gene followed, finally, by a second cassette-unique restriction site, SalI.

E. Construction of Single Cassette Expression Carrier Vectors

Single cassette expression carrier vectors were constructed using pCMC60 as the intermediate carrier vector for the control and replication sequences as follows:

pCMC70 contains coding sequences for a truncated and modified APH-I protein (herein designated mtAPH-I) encoding an aminoglycoside phosphotransferase activity derived from the transposon Tn601 (also known as Tn903) (Sharp, P. A., et al, J Mol Biol (1973) 75:235; Oka, A., et al, J Mol Biol (1981) 147:217). The protein encoded by this sequence is capable of conferring resistance to the antibiotics G418 or kanamycin.

pCMC71 contains the coding sequence for APH-II, an enzyme conferring similar antibiotic resistance, which is encoded on the transposon Tn5 (Jorgensen, R. A., et al, Mol Gen Genet (1979) 177:65).

Also constructed were:

pCMC102 wherein the sequence insert encodes β-interferon.

pCMC103 wherein the insert encodes chloramphenicol acetyltransferase (CAT) which confers resistance to chloramphenicol.

pCMC121 which encodes Bt-toxin, an endotoxin from Bacillus thuringiensis strain HD-1, which is lethal to the larvae of certain insects.

The foregoing are intended, of course, as merely exemplary. The following paragraphs describe the origins of the various coding sequences and their insertion into pCMC60.

E.1 pCMC70

pDG144 contains the modified coding sequence for a truncated, modified APH-I protein (mtAPH-I). The sequence had been modified by destroying the Xma (SmaI) site at the codons for amino acids 93/94 by converting the CCC codon encoding proline (93) to the also proline encoding CCT, and by destroying the HindIII recognition site at amino acids 175/176 by converting the lysine encoding AAG codon to the also lysine encoding AAA. In addition, the N-terminal sequence was truncated and placed immediately downstream from a HindIII recognition site by altering the wild type sequence immediately before the ATG start codon from GGTGTT to AAGCTT, deleting the codons for amino acids 2-10 and fusing the ATG start codon to the codon for amino acid 11. Therefore, the N-terminal sequence immediately preceded by HindIII recognition site now reads AAGCTTATGTCGAGG.

pDG144 was digested with HindIII and PvuII to excise a 1.21 kb fragment terminating in the 3' untranslated sequence of mtAPH-I, and ligated with HindIII and SmaI digested pCMC60 in a two-phase ligation under sticky end and then blunt end conditions. The ligation mixture was then used to transform E. coli MM294 and Amp^R colonies selected and screened for the presence of the desired 5.6 kb recombinant plasmid, pCMC70. The correct construction was confirmed by restriction analysis.

E.2.a. pCMC71

pCMC71 encoding APH-II was constructed similarly. The APH-II gene was obtained by digestion of pAM1 (de Frammond, A., et al, Biotechnology (1983) 1:262-269), a plasmid which confers kanamycin resistance, with BglII and SmaI to yield a 1.0 kb DNA fragment containing

the desired APH-II coding sequence with an additional 36 bp preceding the translation initiation codon. The polylinker sequence in pCMC60 contains BglII and SmaI recognition sites in the correct orientation to accept and express the resulting APH-II fragment, and thus this fragment was ligated with a BglII/SmaI digest of pCMC60, the mixture digested with BamHI to inactivate unwanted fragments and used to transform E. coli MM294. Amp^R and chloramphenicol sensitive colonies were screened for the presence of a 5.35 kb plasmid. pCMC71 was analyzed by restriction analysis and shown to have lost the SmaI site; the remainder of the structure was as expected.

E.2.b. Construction of pCMC72

pCMC72 was constructed from pCMC71 as shown in Figure 5. This construction removes an out of frame ATG codon in the 5'-noncoding region of the APH-II mRNA, and thus results in an elevated level of APH-II protein production relative to pCMC71.

pCMC71 was partially digested with PstI to open a PstI site located within the APH-II coding region, 177 bp from the amino terminal ATG. The plasmid was then digested to completion with BglII, and both the 207 bp BglII/PstI fragment containing the N-terminal coding sequences and a portion of the 5' untranslated region, and the almost full-length vector fragment (missing this 207 bp fragment) were isolated. The 207 bp fragment was then digested with Sau3AI, which removed an additional 24 nucleotides from the BglII end but left a four nucleotide sticky end (GATC) which is compatible with the BglII sticky end. The resulting Sau3AI/PstI fragment was then ligated with the isolated BglII/PstI vector fragment of pCMC71, and transformed into E. coli to Amp^R. The

correct plasmid, pCMC72, was confirmed by restriction analysis.

pCMC72 is identical to pCMC71 except for a 24 bp deletion at the 5' end of APH-II which removes an out of frame ATG codon and the bacterial ribosome binding site.

E.3 pCMC102

pCMC102 contains the β-interferon gene in operable linkage to the components of the carrier cassette. pβ1trp3-4-1, a plasmid containing the β-IFN coding sequence under the control of the trp promoter, was used as a source of the β-interferon gene. It was digested with HindIII and XhoII and the 502 bp fragment containing the mature β-interferon coding sequence was isolated. pCMC60 was digested with HindIII and BglII and the fragments mixed with the purified β-interferon fragment, ligated, and then treated with XbaI to eliminate undesired ligation products. The ligation mixture was used to transform E. coli MM294 and selection made for Amp^R. Successful transformants were screened for the desired 4.89 kb plasmid and the correct construction, pCMC102, was confirmed by restriction analysis.

E.4 pCMC103

The gene encoding chloramphenicol acetyl transferase (CAT) which encodes a protein conferring chloramphenicol resistance was excised from pBR325 (Bolivar, F., et al, Gene (1978) 4:121) by isolating a 775 bp fragment generated by digestion with TaqI. The TaqI fragment was subcloned into the AccI site of the polylinker in pUC13 to give an intermediate plasmid which contains the coding sequence for CAT between a HindIII

site 45 bp upstream of the ATG start codon and an XmaI site 100 bp 3' of the TAA termination codon. The HindIII/XmaI fragment was excised from the intermediate plasmid and purified. pCMC60 was digested with XmaI and HindIII and the linear DNA ligated with the foregoing fragment, followed by BglII digestion to inactivate undesired products. The ligation mixture was transformed into E. coli MM294 and successful colonies were selected for Amp^R. The desired vector, pCMC103, was confirmed by restriction analysis.

E.5 pCMC121

pCMC121, which is a one cassette carrier expression vector for Bt-toxin, was constructed using pCMC60 in a manner analogous to the foregoing constructions. Its construction is outlined in Figure 2.

The recombinant plasmid, pES1 (Wong, et al, J Biol Chem (1983) 258:1960 and Schnepf and Whiteley, Proc Natl Acad Sci (USA) (1981) 78:2893), is a derivative of pBR322 containing as an insert the coding sequences for the Bt-toxin. See also European Application Publication No. 0063949 (November 3, 1982). pES1, in E. coli K12/HB101 is deposited under the terms of the Budapest Treaty at the ATCC under No. 31995. pES1 was mutagenized with the bacterial transposon, Tn5 according to the method of Guyer, M. S., et al, Methods Enzymol (1983) 101:362 to insert a XhoI recognition sequence 5' to the Bt-toxin. A 5.8 kb XhoI fragment was isolated from the pES1-Tn5 plasmid and inserted into the SalI site of pUC9. The ligation mixture was used to transform E. coli K12 JM83 (available from BRL) and the successful transformants selected by Amp^R and lac-. Successful transformants were screened for the presence of the

desired 8.6 kb plasmid, and the construction of the desired pSYC823 confirmed by restriction analysis.

pSYC823 was further modified to place the appropriate restriction sites at the termini of the coding sequences. To create a HindIII site at the 5' terminus, pSYC823 was digested to completion with NdeI, and blunt ended with Klenow in the presence of dATP and dTTP. After complete digestion with HindIII, the DNA was ligated into the phage vector M13mp8 which had been digested with SmaI and HindIII. The desired phage (Intermediate I) containing the 1.8 kb NdeI to HindIII amino-terminal region of the Bt-toxin was identified by miniprep DNA analysis (Holmes, D. S., et al, Anal Biochem (1981) 114:193). The single-stranded DNA form of the desired phage was modified to obtain a HindIII site just 5' of the ATG initiation codon by primer directed mutagenesis, as described by Moller, M. F., et al, Nucleic Acids Res (1982) 10:6487, using the primer

5' GAGGTAA CTTATGG 3' (Bt-toxin sequence)
3' CTCCATTCGAATACC 5' (primer)
HindIII

which contains a 1 base insertion mismatch for the sequence 5' of the ATG start codon and a HindIII site as shown. The resulting modified vector was designated mp8BtRF.

A 3' HindIII site was placed adjacent the Bt-toxin coding region in pSYC823 as follows: Another sample of pSYC823 was partially digested with NdeI, completely digested with PstI, and blunt ended using Klenow in the presence of all four dNTPs. The 7.0 kb fragment was purified, self-ligated, and then transformed into E. coli MM294. The desired intermediate plasmid is

designated pCMC120 and represents pSYC823 with a 1.6 kb deletion of Bacillus sequences from the unique PstI site of the vector to the NdeI site at the 3' end of the Bt-toxin coding region. This deletion placed the HindIII site of the pUC9 vector directly adjacent the 3' end of the toxin coding region and destroyed the PstI site.

The Bt-toxin coding sequence was inserted into the pCMC60 intermediate vector in two portions, the 5' portion from the mp8BtRF 5' HindIII modified segment, and the 3' portion from the pCMC120 3' HindIII modified Bt-toxin coding sequence of the intermediate plasmid above.

mp8BtRF was digested to completion with HindIII and SstI and the 1.4 kb fragment isolated. The second fragment was obtained by digesting pCMC120 to completion with SstI, then partially with HindIII and purifying the 2.3 kb DNA fragment from the HindIII site of the pUC9 vector portion to the SstI site located within the gene. The two foregoing fragments were ligated with HindIII digested, BAPed pCMC60, and transformed into E. coli MM294. Amp^R colonies were selected and screened for the presence of pCMC121, which contains the entire coding sequence for Bt-toxin under the control of the NOS promoter and polyadenylation signal sequences.

E.6 Construction of an Intermediate and Expression Carrier Plasmid Suitable for Expression of a Fusion Protein

pCMC101 is analogous to pCMC60 except that, instead of the NOS promoter which terminates in a convenient HindIII site suitable for the insertion of a mature coding sequence, pCMC101 has a chimaeric segment with the NOS promoter fused to coding sequences for β-galactosidase. Subsequent insertion of desired coding

sequence in reading frame with the β-galactosidase codons results in a fusion protein containing both β-galactosidase sequences and amino acid sequences corresponding to the inserted codons. It is also an expression carrier vector in that it is capable of effective expression of the NOS-β-galactosidase fusion. The construction is outlined in Figure 3.

Plasmid pMC1403 (Casadaban, M. J., et al, J Bacteriol (1980) 143:971) contains the β-galactosidase coding sequence with a polylinker at the 5' end. To provide a convenient 3' terminal restriction site, the lac-Z portion of pMC1403 was excised by digestion with EcoRI (which cleaves in the polylinker) and ClaI which cleaves just 3' of the lac-Z gene (Buchel, D. E. , et al, Nature (1980) 283:541). The EcoRI/ClaI 3.4 kb fragment was cloned into EcoRI/ClaI digested pOG2326, a bifunctional replicon capable of replication in both E. coli and B. subtilis and derived from pBR322, to obtain pDH5425. pDH5425 was digested with BamHI to obtain a 3.8 kb fragment spanning the BamHI site of the polylinker sequence at the lac-Z 11th codon to the BamHI of the Tet^R gene of the vector plasmid. The BamHI fragment was subcloned into the BamHI site of pUC13 to give pCMC9163 which thus has a 5' terminal polylinker on the lac-Z gene as follows:

```
amino acid #:      9   10   11   12   13
                   ___  ___  ___  ___  ___
5' ACT CTA GAG GAT CCC-----lacZ-3'
   XbaI     BamHI
```

To construct the chimaeric promoter/coding sequence, the pCMC29 intermediate plasmid (paragraph D.2), which contains the unmodified NOS promoter, was

used. This plasmid has a polylinker region following the codon 16 of the original NOS coding sequence as follows:

amino acid #: 16 17* 18* 19* 20* 21* 22* 23* 24* 25* 26*

5'- GAT CCT CTA GAG TCG ACC TGC AGC CCA AGC TTG

Sau3AI/BamHI XbaI HindIII

fusion

where * represents codons substituted for the native NOS sequence.

The XbaI sites of pCMC29 and pCMC9163 are in identical reading frames. Further, there is a unique HindIII site at the 3' end of the polylinker in pCMC29 and at the 3' end of the β-galactosidase coding sequences in pCMC9163. Therefore, both plasmids were digested to completion with HindIII and XbaI, the fragments ligated, and the ligation mixture was transformed into E. coli MM294. Successful colonies were selected for Amp^R.

The resulting plasmid, pCMC17, thus comprises the restriction sites EcoRI, SstI, SmaI(XmaI) of the pUC13 polylinker, the NOS promoter and the amino terminal coding region for NOS fused in correct reading frame to the lac-Z gene, the coding sequences for β-galactosidase downstream from codon 18, followed by a HindIII site.

To complete the construction of the intermediate (and expression) carrier vector pCMC101 containing the chimaeric NOS/lac-Z sequences, pCMC60 was digested with EcoRI and HindIII and the vector fragment ligated with the 3.4 kb fragment obtained from pCMC17 by digestion with EcoRI and HindIII containing the NOS promoter and amino terminal codons and fused lac-Z gene. The resulting vector, pCMC101, was obtained from successful E. coli MM294 transformants selected for Amp^R

by screening for the desired 7.9 kb plasmid and confirmed by restriction analysis.

F. Construction of Double Cassette Carrier Vectors

Because each of the expression carrier vectors has convenient cassette-unique restriction sites, both 5' and 3' of the expression cassette, these cassettes can be recombined into multiple cassette vectors in a straightforward manner. Since the coding sequence in pCMC71 for APH-II results in a selectable marker characteristic, pCMC71 was used as a recipient vector for the expression cassettes of the other carrier expression vectors described above. Of course, interchange could be made between any of the various carrier vectors constructed.

pCMC77 contains a double cassette which includes the APH-II coding sequence and the β-interferon sequence from pCMC102. To construct this vector, pCMC102 was digested to completion with XhoI and SalI, and the desired 1.1 kb DNA fragment purified. The fragment was ligated with SalI digested, BAPed pCMC71, and the ligation mixture was transformed into E. coli MM294. Amp^R colonies were screened for the presence of the desired 6.5 kb plasmid, pCMC77. The correct construction was confirmed by restriction analysis. As the ligation of XhoI and SalI sticky ends does not regenerate either a SalI or XhoI site, the resulting vector has XhoI and SalI sites which remain "cassette-unique" at the 5' and 3' termini of the series, if the donor expression cassette is unidirectional with the recipient vector DNA sequences; the SalI site is between the expression cassettes if the opposite orientation results (see paragraph B.2 above).

Similar XhoI/SalI digestions were used to prepare the CAT cassette from pCMC103, the mtAPH-I cassette from pCMC70, and the Bt-toxin cassette from pCMC121. These fragments were ligated into SalI digested pCMC71 to form the double-cassette expression carrier vectors below:

pCMC78 containing the APH-II and CAT cassettes,
pCMC79 containing the APH-II and mtAPH-I cassettes,
pCMC75, containing the APH-II and Bt-toxin cassettes,

The NOS-lac-Z fusion cassette was obtained from pCMC101, because there was no XhoI site 5' to the gene, by digestion instead with EcoRI (a 5' cassette-unique site), and SalI. This 5' EcoRI/3'SalI galactosidase chimaera was ligated into pCMC71 which had been digested with EcoRI and XhoI to give a double cassette recombinant plasmid (pCMC76), selected following minipreps.

G. Construction of Single Cassette and Double Cassette Expression Carrier Vectors Adaptable to Agrobacterium Mediated Transformation

pCMC90, pCMC91 and pCMC92 are single cassette expression vectors for the mtAPH-I or APH-II genes framed by border sequences and having a broad host range bacterial origin of replication. These constructions are outlined in Figure 4. As shown in Figure 4, pCMC91 is constructed from pCMC90 by replacing the mtAPH-I coding region with the APH-II coding sequence from pCMC71. pCMC92 is formed from pCMC90 using pCMC72 instead of pCMC71. These plasmids are suitable for use in Agrobacterium mediated transformations.

G.1 Construction of pCMC90

pCMC90 was constructed from the intermediate vectors described below by inserting a 2.8 kb EcoRI/SalI cassette from pCMC80, which contained the mtAPH-I coding sequences with suitable control segments along with right border (RB) T-DNA into a broad spectrum replicating host plasmid bearing the left border (LB) regions, pCMC25.

G.1.a. Construction of pCMC80

pCMC70, prepared as described in paragraph E.1, is a carrier expression vector for the modified, truncated (mt) dominant selectable marker, mtAPH-I. It contains the NOS promoter as an EcoRI/HindIII cassette immediately preceding the coding sequence. In pCMC80, this cassette is replaced by a larger EcoRI/HindIII cassette, which cassette contains the RB sequence from pTiT37 along with the NOS promoter modified to have a HindIII site at the 3' end of the promoter as in pCMC70 preceding the site for translation initiation as in pCMC70.

pCMC1 was digested with HindIII, treated with PolI Klenow fragment in the presence of dNTPs, and then digested with SstII. The 1.1 kb HindIII(blunt)/SstII fragment was isolated and ligated with DNA fragments obtained by digesting pCMC70 with XhoI, treating with Klenow and the dNTPs, and then digesting with SstII. Approximately 200 ng of the DNA from the ligation mixture was used to transform E. coli MM294 to Amp^R.

Successful colonies were screened for the desired 6.55 kb recombinant plasmid pCMC80, which contains the Ti T-DNA RB, the NOS promoter, the mtAPH-I coding sequences, and the NOS polyadenylation signals followed by a SalI site. Although the fusion of PolI

treated XhoI and HindIII sites would have been expected to regenerate a HindIII site, the selected plasmid pCMC80 had lost this site. The foregoing sequences are available as a 2.8 kb EcoRI/SalI DNA fragment, the 5' XhoI site of pCMC70 having been lost. Thus, pCMC80 exemplifies a carrier expression vector for a foreign gene with a narrow host range baterial origin of replication, containing a right border sequence properly placed with respect to the transcription cassette.

G.1.b. Construction of pCMC25

pCMC25 was constructed as follows: The LB sequence was obtained from pTiT37 by digesting with EcoRI and isolating the 1.6 kb fragment 29 using standard methods. This fragment was cloned into the EcoRI site of pAM1, a derivative of pBR325 in which a 1.5 kb HindIII/SalI DNA fragment from transposon Tn5 containing the APH-II antibiotic resistance gene had been substituted for the HindIII/SalI region of the Tet^R gene.

The ligation mixture containing the thus modified pAM1 vector was cloned into E. coli MM294 and selected for Amp^R. Successful colonies were screened for the desired 8.1 kb plasmid, pCMC5, which contained the LB region of T-DNA contiguous with the APH-II gene.

pCMC5 was further modified to provide a broad spectrum bacterial origin of replication by utilizing the vector portions of RSF1010 (Bagdasarian, M., et al, Gene (1981) 16:237), a broad host range, multicopy plasmid containing a sulfonamide resistance (Su^R) gene and capable of replicating both in E. coli and in A. tumefaciens. To do this, pCMC5 was partially digested with BglII and then further digested with SalI to produce the desired 3.4 kb BglII/SalI fragment containing the T-DNA LB sequence and adjacent APH-II

gene. The fragment was repaired with Klenow and dNTPs to provide a blunt-ended fragment having the sequence 5'-GATCT-LB-APH-II-GTCGA-3'. This blunt-ended fragment was ligated with SstI digested, Klenow repaired RSF1010 which thus provided a vector sequence with 5' C and 3' G terminal nucleotides. The ligation mixture was transformed into E. coli MM294 and successful transformants selected by Kan^R Su^R and successful transformants screened for the appropriate 12.7 kb plasmid. Correct construction of pCMC15 was confirmed by restriction analysis: the desired construction contains a single SalI site at the point of ligation of the APH-II DNA but has lost the SstI and BglII sites.

pCMC15 was further modified to insert a polylinker region adjacent the T-DNA-LB segment. The polylinker was derived from pUC13 by digestion with EcoRI, repaired with Klenow and all four dNTPs, followed by further digestion with SalI. This provided a 32 bp EcoRI(blunt)/SalI fragment containing SstI, SmaI(XmaI), BamHI, XbaI, and SalI sites, which was isolated by standard methods. This fragment was ligated with an isolated 10.2 kb fragment produced by partial digestion of pCMC15 with EcoRI to open the EcoRI site between the LB and APH-II, repairing with Klenow, and further digesting with SalI. The ligation mixture was transformed into E. coli MM294 to Su^R and subsequently transformants were screened for kanamycin sensitivity. The transformants were screened for the presence of the desired 10.2 kb plasmid, pCMC25, which contains a polylinker region proximal to the LB sequences.

G.1.c. Completion of pCMC90

The desired pCMC90 was constructed as follows: pCMC25 was digested with with EcoRI and SalI

to open the vector next t the polylinker region. pCMC80 was digested with EcoRI and SalI to release the mtAPH-I expression cassette which was purified and ligated with the opened pCMC25 vector. The ligation mixture was used to transform E. coli MM294 to Su^R, and Amp^S transformants were screened for the presence of the desired 12.9 kb expression carrier plasmid, pCMC90.

To summarize, pCMC90 is a multicopy, Su^R Amp^S derivative of the wide host range plasmid, RSF1010, which contains an expression cassette for the modified mtAPH-I sequence bordered by the T-DNA right and left border sequences. The inserted cassette contains a unique SalI site at its 3' end between the polyadenylation signal and the LB sequence.

G.2. Construction of pCMC91

To obtain pCMC91, pCMC90 was digested with HindIII and SalI, and the approximately 11.5 kb vetor fragment was purified. pCMC71 was digested with HindIII and SalI, and the 1.3 kb fragment containing the APH-II coding region and NOS termination region was also purified. The 11.5 kb vector and 1.3 kb APH-II fragments were then ligated and transformed into E. coli MM294 to Su^R. The correct construction of pCMC91 was confirmed by restriction analysis and pCMC91 was deposited with ATCC on or about 10 April 1985 and has accession no. _____.

G.3. Construction of pCMC92

pCMC92 was constructed in a manner exactly analogous to that described for pCMC91 except that pCMC72 was used instead of pCMC71 as the source of the APH-II gene. Thus, pCMC92 has a modified 5' untranslated region for the APH-II gene lacking the out of frame ATG and the

bacterial ribosome binding site. pCMC92 was deposited with ATCC on or about 10 April 1985 and has accession no. _____.

G.4. Construction of Double-Cassette, Broad Host Range-T-DNA-Bordered Expression Carrier Vectors

pCMC112 contains a T-DNA bordered double expression cassette for APH-II and β-IFN on a vector with a broad host range replicon. It was prepared by digesting pCMC91 with SalI, treating with BAP, and ligating the linearized DNA with the 1.1 kb fragment isolated after XhoI/SalI digestion of pCMC102. The ligation mixture was transformed into E. coli MM294, and Su^R colonies screened for the desired 13.8 kb pCMC112. It contains a unique SalI site between the IFN cassette and the LB sequence.

In a precisely similar manner, XhoI/SalI expression cassettes were excised from the expression carrier vectors, pCMC70 (paragraph E.1), pCMC103 (paragraph E.4), and pCMC121 (paragraph E.5), and ligated into the SalI site of pCMC91. Thus were produced the following double cassette expression carrier vectors adaptable to transformation mediated by Agrobacterium:

pCMC114 containing the APH-II and mtAPH-I cassettes;
pCMC113 containing the APH-II and CAT cassettes;
pCMC123 containing the APH-II and Bt-toxin cassettes.

pCMC123 was deposited with ATCC on or about 10 April 1985 and has accession no. _____.

In all of the foregoing, the cassette series is framed by right border and left border T-DNA sequences, resides on

a plasmid containing a broad host range origin of replication, and in most cases contains a cassette-unique (SalI) site between the polyadenylation signal of the cassette series and the LB sequence. However, as described in B.2, the opposite orientation of the donor cassette will result in the placement of the SalI site between the expression cassettes.

Also, an EcoRI(blunt)/SalI cassette can be isolated from pCMC101 (paragraph E.6) and ligated into SalI/SmaI digested pCMC91. Alternatively, pCMC101 can be digested with SalI, and this plasmid can be ligated with SalI digested pCMC91, generating a cointegrate plasmid (pCMC91/101) in which the entire galactosidase expression vector is inserted into the vector, pCMC91.

The following examples illustrate the use of the expression carrier vectors prepared above and the results therefrom. In paragraph H is illustrated the direct transformation of plant cells using pCMC71, pCMC91, and pCMC77. Of course, any of the other vectors could as well have been used in the same fashion.

Paragraph I illustrates the use of pCMC91, pCMC111, and pCMC123 in Agrobacterium-mediated transformation. All of the vectors whose construction was illustrated in paragraph G above could as well have been used.

H. Direct Transformation Using pCMC71, pCMC91, and pCMC77

The procedure of this paragraph was used to transform plant cells with pCMC71, pCMC91, and pCMC77. As used in this paragraph, "expression carrier vector" refers to each of these three plasmids.

N. tabacum var. H425 protoplasts were prepared from sterile shoots as described by Krens, et al, Nature

(1982) 296:72, and suspended in a K_3 medium containing 0.4 M sucrose, naphthalene acetic acid (NAA) at 0.1 mg/l and kinetin at 0.2 mg/l. The protoplasts (5×10^5 cells in 1 ml) were combined with 0.5 ml of 40% (w/v) polyethylene glycol 6000 dissolved in an uptake buffer (UB) consisting of 140 mM NaCl, 5 mM NaCl, 5 mM KCl, 0.75 mM Na_2PO_4, 5 mM glucose and 125 mM $CaCl_2 \cdot 2H_2O$ at pH 7.0. To this mixture was added 50 µl solution containing 1 to 10 µg expression carrier vector and 0 to 50 µg of purified N. tabacum var. H425 DNA, added to protect against degradation. (Higher rates of transformation were observed in the presence of the purified plant DNA.)

The protoplasts were incubated at 25°C for 30 min with very gentle shaking. At 5 min intervals thereafter, 2 ml aliquots of the UB medium were added dropwise to the solution, until a total of 10 ml new UB medium had been added. The protoplasts were pelleted by low speed centrifugation and the supernatant was removed. The pellet was resuspended in 10 ml K_3 medium and plated on sterile Whatman #2 (qualitative) filter paper discs, and separated from a feeder layer of wild type N. tabacum var. H425 suspension cells by an additional layer of filter paper. The cells were grown on a standard M3 nutrient medium (Marton, et al, Nature (1979) 277:129) containing phytohormones.

Cells were plated at a density of 10^2 to 10^4 per 10 cm Petri plate. The cells were incubated in the dark for 24 hr and then grown for 2 weeks at 2,000 lux in 12 hr photoperiods at 25°C. Cell survival was approximately 50% after this time period. The upper filter layer on which small cell clumps were grown was then transferred to M3 medium supplemented with phytohormones (0.1 mg/l NAA and 0.2 mg/l kinetin) and G418 (25 mg/l) or kanamycin (100 mg/l) and incubation

was continued at 25°C with 12 hr photoperiods. Alternatively, transformed cells were plated at a density of 10^5/ml in T-75 culture flasks. Cells grow effectively into individual small clumps in two weeks, and such calli were plated at desired densities onto selective media. Calli resistant to the antibiotic continued to expand, while those apparently not transformed by plasmid marker DNA APH-II ceased growth and gradually turned brown over the next 2 to 4 weeks.

When the calli grown under the above conditions had reached approximately 1 mm in diameter, they were harvested and regenerated into plantlets as follows: calli were picked by scalpel point and placed on M3 medium solidified with 0.4% agar containing phytohormones and kanamycin (100 mg/l). When these calli reached 2 mm or more in diameter, small pieces were transferred to M3 medium containing 0.1 mg/l NAA and 0.5 mg/l to 1 mg/l kinetin to induce shoot formation. Shoots were then excised and placed in rooting medium (1/10 strength M3 medium without sucrose or phytohormones, 0.4 mg/l thiamine and 1% agar, pH 7.9, as described by Barton, K., et al, Cell (1983) 32:1033). Rooted plantlets were placed in soil under high humidity for several days, followed by routine greenhouse growth conditions.

H.1 Results from Transformation with pCMC71

H.1.a. Insertion of DNA into the Genome

High molecular weight DNA is isolated from young leaves of plants prepared as in paragraph H as described by Chilton, M. D., et al, Nature (1982) 295:432. Following banding of the DNA in CsCl-ethidium

bromide gradients, dye is extracted with isopropyl alcohol equilibrated with 3M NaCl, 0.3 M sodium citrate. The clear aqueous phase is diluted with 3 volumes of 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, and DNA precipitated by the addition of absolute ethanol to a final concentration of 70%.

DNA from transformed and non-transformed plant cells and from cassette vector pCMC71 (as a control) are digested to completion with HindIII and SalI. Standard pCMC71 digests are carried out in the presence of 5 µg calf thymus DNA per well as carrier. The digested DNAs are loaded into 1x8x8 mm wells in a horizontal 0.6% agarose gel prepared in Tris-acetate buffer (Chilton, M. D., et al, Cell (1977) 11:263). Agarose-gel electrophoresis and transfer of gel-fractionated DNA to nitrocellulose are carried out as described in Thomashaw, et al, Proc Natl Acad Sci (USA) (1980) 77:6448. Plant DNA samples are loaded at 5 µg per well, and the pCMC71 DNA at various concentrations corresponding to 1 to 100 genome equivalents.

Hybridization probes are generated by nick-translation of purified HindIII/SalI 1.3 kb DNA fragment from plasmid pCMC71, according to the general method of Maniatis, T., et al, Proc Natl Acad Sci (USA) (1975) 72:1184. The probe is used at a specific activity of approximately 10^8 cpm/µg DNA for Southern blot analysis of nitrocellulose-bound DNA fractions (Chirgwin, T., et al, Biochemistry (1979) 18:5294). Results of the Southern analyses show the presence of a HindIII/SalI 1.3 kb DNA gene fragment in DNA from regenerated, transformed plants, corresponding in size to the DNA fragment from pCMC71 which encodes the APH-II protein. No similarly sized DNA fragment is observed in the DNA of normal (untransformed) tobacco plants.

H.1.b. Transcription of pCMC71

Total RNA fractions from young transformed and normal plants are isolated according to the method of Chirgwin (supra), as modified by Bevan, et al, J Mol Appl Genet (1982) 1:539. Leaves and stems of young plants (5 to 50 grams per extraction) are frozen in liquid nitrogen, powdered, and lyophilized until dry. The tissue is homogenized in extraction solution containing 4 M guanidine thiocyanate, 25 mM Tris-Cl, pH 7.0, 0.5% sarkosyl and 0.03% Antifoam A Sigma. Sarkosyl is a trademark for sodium lauryl sarcosinate and was obtained from Sigma. The homogenate is prepared to a concentration of 1 gram initial tissue weight/ml extraction solution. The homogenate is filtered through cheesecloth, followed by centrifugation at 5000 x g for 10 min to remove particulate matter, and the supernatant liquid adjusted to pH 5.0 with 1 M acetic acid; one volume absolute ethanol is added to precipitate nucleic acids. After 2 hr at -20°C, the precipitate is pelleted by centrifugation at 5000 x g for 30 min and redissolved in 25 ml of 7.5 M guanidine hydrochloride, 25 mM Tris-Cl, pH 7.0. The pH is adjusted to 5.0 with 1 M acetic acid, and 18 ml absolute ethanol added. The precipitate which forms after about 12 hr at -20°C is collected by centrifugation, washed twice with 70% ethanol, and dissolved in 25 ml of 25 mM EDTA, 0.1% diethyl pyrocarbonate, pH 8.0. This solution is extracted with water saturated phenol:chloroform mixture (6:5) and precipitated with 2 volumes ethanol. The pellet is then resuspended in 8 ml 25 mM Tris-Cl, pH 8.0, containing 0.1% diethylpyrocarbonate. Two ml of 10 M LiCl is added to precipitate RNA selectively, and the RNA pellet resulting from centrifugation is fractionated by oligo-dT

cellulose chromatography (Bantle, et al, Anal Biochem (1978) 72:413.

Polyadenylated RNA (polyA RNA) is denatured with 1 M glyoxal at 50°C for 1 hr (McMaster, et al, Proc Natl Acad Sci (USA) (1977) 74:4835). The RNA is electrophoresed and subjected to Northern blot analysis, as described by Bevan, et al, J Mol Appl Genet (1982) 1:539. Nick translated DNA prepared as in paragraph H.1.a and labeled to a specific activity of approximately 10^8 cpm per µg, is used to probe for APH-II mRNA. Tobacco ribosomal RNAs are used as molecular weight standards on gels.

The pCMC71 probe shows no hybridization to polyA RNA in normal (untransformed) tobacco, but a hybridization band is observed in mRNA isolated from plants regenerated from pCMC71-transformed tissue cultures. The estimated size of the APH-II mRNA is in excess of 1000 nucleotides.

H.1.c. Translation of APH-II in Transformants

Effective selection for G418 (or kanamycin) resistance to the transformed plant cells implies that the introduced gene is expressed. This is confirmed by showing the presence of APH-II protein in transformed plant cells by immunoassay.

Tissue samples from the regenerated plants are grown under conditions of brief radioactive amino acid labeling with ^3H glycine. Plant supernatant proteins are extracted by the method of Barton, K., et al, J Biol Chem (1982) 257:6089. Supernatant proteins (500 µg/ml) from either transformed or normal plants, are mixed with 0.1 volumes of either pre-immune or immune, anti-APH-II rabbit serum prepared by standard

procedures. Immunopecipitation is carried out at 37°C for 4 hr. The immunoprecipitates are pelleted by centrifugation, dissolved in sodium dodecyl sulfate (SDS) and analyzed by SDS-PAGE. The radioactive banding pattern was determined by fluorography (Laskey, R. A., et al, Eur J Biochem (1975) 56:335).

Minor radioactive bands of low molecular weight (less than 27,000 daltons) are present in both normal and transformed plants, using both pre-immune serum as well as anti-APH-II serum. Distinct bands of radioactivity are observed at approximately 27,000 daltons, corresponding to the apparent molecular weight of APH-II in the supernatant proteins derived from transformed cells, but not in the supernatant from untransformed plant tissue. This polypeptide was immunoprecipitated only with anti-sera prepared against APH-II.

H.2 Results of Transformation with pCMC91

As the coding sequences to be inserted using pCMC91 are identical to those inserted using pCMC91, precisely the same methods are employed and the same results are obtained in confirming the integration of the DNA into the genome, the transcription of the coding sequence, and the translation of the messenger.

H.3 Results of Transformation with pCMC77

Successful transformation and integration into the genomic DNA from the double cassette vector, pCMC77, is confirmed in an analogous manner to the foregoing procedures for pCMC71. However, in carrying out the Southern blot analysis according to the procedure of

paragraph H.1.a, the probe is prepared by nick-translating the 0.5 kb HindIII/BglII β-IFN gene fragment from pCMC102, and the DNA extracted from the transformed plant cells digested with both EcoRI and SalI. The double digestion releases the β-IFN gene as a 1.1 kb fragment.

H.4 Integration of Other Genomic Sequences

Similarly, confirmation of integration of coding sequences from other vectors of the invention is obtained by constructing nick translated probes for the coding sequence DNA by suitable digestion of the expression carrier vector with restriction enzymes, and using standard Southern blot techniques to detect the presence of the desired fragment in the correspondingly digested genomic DNA.

I. Agrobacterium Mediated Transformation of N. tabacum

All of the expression carrier vectors of the invention which contain the T-DNA border sequences, which mediate Agrobacterium assisted transformation, can be used in the procedures of this paragraph I. Construction of such vectors is illustrated in paragraph G, above. The description of this paragraph is set forth in terms of pCMC91, however, any of the described vectors or other vectors of the invention which contain T-DNA border sequences and broad host bacterial origins of replication can be used. The procedure illustrates the use of an Agrobacterium mutant which contains a disarmed Ti plasmid to supply the virulence regions. However, alternate means of supplying such sequences, absent the tumor-producing sequences in the T-DNA region of the Ti plasmid, such as cotransformation of an "empty"

Agrobacterium strain with both a disarmed Ti plasmid and an expression carrier vector of the invention could be used.

I.1. Transformation of A. Tumefaciens with pCMC91

The disarmed Ti vector employed was that harbored by A. tumefaciens strain LBA4404, available from Dr. Paul Hooykaas at the University of Lyden (Ooms, G., Hooykaas, P. J. J., et al, Gene (1981) 14:33). This strain is non-oncogenic as it harbors only pAL4404, an avirulent deletion mutant derivative of the octopine Ti plasmid, pTiAch5, which has a Tn904 insert in SmaI fragment 3A, and has suffered a deletion extending from this point rightward to SmaI fragment 6. This plasmid thus lacks the entire octopine T-DNA and octopine metabolism functions, but retains an intact virulence region on the left side of the T-DNA (See de Framond, A. J., et al, Biotechnology (May 1983) 262-269; Hoekema, A., et al, Nature (1983) 303:179). A. tumefaciens harboring both pCMC91 and the disarmed Ti plasmid pAL4404 was obtained by transforming A. tumefaciens strain LBA4404 with pCMC91 by the freeze-thaw method of Holsters, et al, Mol Gen Genet (1978) 163:181, and selecting for Su^R.

I.2. Transformation of N. tabacum Stem Segments with Agrobacterium-Harboring pCMC91 and the Disarmed Ti Plasmid

Stems of Nicotiana tabacum, variation Havana 425 (H425) were surface sterilized with 7% commercial Chlorox and 80% ethanol, then rinsed with sterile distilled water and cut into 1 cm long segments. The segments were placed basal end up in Petri dishes containing complete MS medium (Binns, A., et al, Planta

(1979) 145:365) with hormonal supplements of 2 mg/l naphthalene acetic acid (NAA) and 0.3 mg/l kinetin. The basal end of each stem segment was punctured repeatedly with syringe needles and inoculated with A. tumefaciens strain LBA4404 transformed with pCMC91. After 5 to 8 days incubation of the inoculated stem segments at 25°C, with 16 hr of light each day, calli developed at various positions on the stem segments. The callus regions were removed from the stem segments and transferred to complete MS medium (MS basal medium supplemented with 2.0 mg/l NAA and 0.3 mg/l kinetin). The medium also contained 200 mg/l carbenicillin and 500 mg/l vancomycin to kill the inoculating bacteria, and antibiotics to select for transformed plant cells. The selection of antibiotics routinely included kanamycin (100 mg/l) or G418 (25 mg/l) although neomycin or lividomycin are also acceptable alternatives. After three transfers (at approximately 4-week intervals) of the small clumps of viable cells which were resistant to kanamycin or G418, the calli are sufficiently enriched in transformed cells relative to non-transformed cells to enable assay for the presence of heterologous DNA in the cells, transcription of the heterologous gene, and heterologous-DNA gene expression.

When the calli grown under the above conditions reach approximately 1 mm in diameter, they are picked by scalpel point and placed on M3 medium solidified with 0.4% agar containing phytohormones and kanamycin (100 mg/l). Once calli reaches 2 mm or more in diameter, small pieces are transferred to M_3 medium containing 0.1 mg/l NAA and 0.5 mg/l to 1 mg/l kinetin to induce shoot formation. Shoots are then excised and placed in rooting medium (1/10 strength MS media without sucrose or phytohormones, 0.4 mg/l thiamine and 1% agar, pH 7.0). Rooted

plantlets are placed in soil under high humidity for several days, foll wed by r utine greenhouse growth conditions.

I.3 Co-cultivation Transformation with pCMC91 and Disarmed Ti Plasmid

N. tabacum variation H425 seedlings were grown under sterile conditions and young leaves were treated to generate viable protoplasts, as described in paragraph H.1. The protoplasts were cultured in a K_3 medium supplemented with NAA at 0.1 mg/l and kinetin at 0.2 mg/l for 24 hr in the dark, followed by 48 hr incubation at 2,000 lux. The cells, with regenerating cell walls, were mixed with A. tumefaciens LBA4404 transformed with pCMC91 at 10^5 plant cells and 10^7 bacterial cells per ml.

The cells were incubated in K_3 medium supplemented with phytohormones as above for approximately 24 hours at 20°C at 2,000 lux, after which time bacteria were removed by repeated washing, with the final wash in K_3 medium supplemented with phytohormones as above, and 200 mg/l carbenicillin and 250 mg/l vancomycin. Cells were then placed on Whatman #2 filters over feeder layers (as described above for single cell cloning) in M_3 medium containing phytohormones, carbenicillin at 200 mg/l and vancomycin at 250 mg/l. Plant cell densities were varied between 10^2 and 10^5 cells per 10 cm Petri plates, the higher dilutions giving rise to colonies resulting from a single cell. Cells were allowed to form calli for up to 4 weeks on such feeder plates, until clumps of cells approximately 0.5 mm in diameter were visible. The upper filters were then transferred to fresh plates of M_3 medium containing carbenicillin at 200 mg/l and kanamycin at 100 mg/l (or alternatively G418 at 25 mg/l). Under this

procedure, transformed calli were rapidly selected by continued growth due to resistance to the antibiotic kanamycin, while non-transformed calli ceased growth and turned brown. After calli reached 1 to 2 mm in diameter, the calli are transferred to fresh plates and increased in number of cells for direct regeneration of plants as described in paragraph I.2 and I above.

I.4. Results of Transformation

Successful transformations are confirmed in precisely the same manner as set forth in paragraphs H.1.a-H.1.b for the transformation of plant cells with pCMC71. Of course, the results are identical as well. Similarly, using the same procedures but the appropriate probes and antibodies, successful transformations using any gene sequence encoding cassette can be monitored.

The following plasmids have been deposited at the American Type Culture Collection, Rockville, Maryland, U.S.A. (ATCC) under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder (Budapest Treaty) and are thus maintained and made available according to the terms of the Budapest Treaty. Availability of such strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The deposited plasmids have been assigned the indicated ATCC deposit numbers. The plasmids have also been deposited with the Master Culture Collection (CMCC) of Cetus Corporation, Emeryville, California, U.S.A., the assignee of the present application, and assigned the indicated CMCC deposit numbers:

Plasmid and Host	CMCC Deposit No.	ATCC Deposit No.
pβltrp3-4-1 in E. coli K-12/MM294	1730	39646
pCMC1 in E. coli K-12/MM294	1985	39641
pCMC15 in E. coli K-12/MM294	2002	39654
pDG144 in E. coli K-12/MM294	1960	39579
pD85425 in E. coli K-12/MC1000	1503	39645
pOG2326 in E. coli K-12/CS412	1577	39600
pSYC823 in E. coli K-12/JM103	2020	39657
pTiT37 in A. tumefaciens A208	1773	39423
pCMC123 in E. coli K-12/MM294		
pCMC91 in E. coli K-12/MM294		
pCMC92 in E. coli K-12/MM294		

Claims

1. An intermediate carrier vector which comprises a DNA sequence cassette containing, in order, proceeding 5'-3' in the sense strand:

(a) a first cassette-unique restriction site;

(b) a promoter sequence (oriented 5'-3') which is normally operable in plant cells;

(c) a polylinker;

(d) a polyadenylation signal (oriented 5'-3') operable in plant cells; and

(e) a second cassette-unique restriction site.

2. The vector of claim 1 which further includes a desired coding sequence contained in the polylinker of (c) operably linked to the promoter of (b) and the polyadenylation signal of (d).

3. The vector of claim 1 which further includes a vector sequence comprising a broad host range bacterial origin of replication.

4. The vector of claim 3 which further includes a T-DNA border sequence proximal to either or both of the first restriction site of (a) and to the second restriction site of (e).

5. An expression carrier vector which comprises an expression cassette containing:

(a) a promoter sequence normally operable in plant cells, operably linked to

(b) a desired coding sequence;

(c) a polyadenylation signal operable in plant cells, operably linked to the desired coding sequence of (b), and

(d) a cassette-unique restriction site proximal to a cassette terminus.

6. The vector of claim 5 wherein the expression cassette has a cassette-unique restriction site proximal to each cassette terminus.

7. The vector of claim 5 which further includes a vector sequence comprising a broad host range bacterial origin of replication.

8. The vector of claim 7 which further includes a T-DNA border sequence proximal to either or both of the expression cassette termini.

9. An expression carrier vector which comprises a DNA sequence cassette containing:

(a) a first promoter sequence normally operable in plant cells, operably linked to

(b) a first desired coding sequence;

(c) a first polyadenylation signal operable in plant cells, operably linked to the first desired coding sequence;

and at least one additional expression sequence comprising:

(d) a second promoter normally operable in plant cells, operably linked to

(e) a second desired coding sequence;

(f) a second polyadenylation signal operable in plant cells, operably linked to the desired coding sequence; and

(g) a cassette-unique restriction site proximal to a DNA sequence cassette terminus.

10. The vector of claim 9 which further includes a vector sequence comprising a broad host range bacterial origin of replication.

11. The vector of claim 10 which further includes a T-DNA border sequence proximal to either or both of the DNA sequence cassette termini.

12. An expression cassette operable in transformed plant cell hosts comprising:

(a) a first cassette-unique restriction site;

(b) a promoter sequence normally operable in plant cells, operably linked to

(c) a desired coding sequence;

(d) a polyadenylation signal operable in plant cells, operably linked to the desired coding sequence of (c); and

(e) a second cassette-unique restriction site.

13. An expression carrier vector which comprises an expression cassette containing

(a) a promoter normally operable in plant cells and a polyadenylation signal operable in plant cells, both operably linked to a desired coding sequence, and

(b) at the 5' or 3' terminus of the cassette, a cassette-unique restriction site.

14. An expression carrier vector which comprises an expression cassette containing

(a) a promoter normally operable in plant cells and a polyadenylation signal operable in plant cells, both operably linked to a desired coding sequence, and

(b) at the 5' or 3' terminus of the cassette, a cassette-unique restriction site

said cassette being framed by T-DNA border sequences,

wherein the carrier vector contains a broad host range bacterial origin of replication.

15. A plant, including the cells and seeds thereof, transformed with the vector of claim 5.

16. A plant, including the cells and seeds thereof, transformed with the vector of claim 8.

17. A plant, including the cells and seeds thereof, transformed with the vector of claim 9.

18. A plant, including the cells and seeds thereof, transformed with the vector of claim 11.

19. A method of transforming a plant, including the cells and seeds thereof, which comprises directly transforming a culture of host plant cells with an expression carrier vector comprising a promoter normally operable in plants and a polyadenylation signal operable in plant cells, both operably linked to a desired coding sequence.

20. A method of transforming a plant including the cells and seeds thereof which comprises directly transforming host plant cells with the vector of claim 5.

21. A method of transforming a plant including the cells and seeds thereof which comprises directly transforming host plant cells with the vector of claim 9.

22. A method of transforming plant cells which comprises:

(a) transforming A. tumefaciens with an expression carrier vector which comprises an expression cassette containing a promoter normally operable in plant cells and a polyadenylation signal operable in plant cells, both operably linked to a desired coding sequence, said expression cassette being proximal to at least one T-DNA border sequence; and a vector fragment containing a broad host range bacterial origin of replication;

(b) providing in the A. tumefaciens host a plasmid comprising the virulence region of a Ti plasmid; and

(c) contacting susceptible plant cells with the resulting A. tumefaciens.

23. A method of transforming plant cells which comprises contacting A. tumefaciens-susceptible plant cells with cells of A. tumefaciens which contain:

(a) plasmid comprising the virulence region of a Ti plasmid;

(b) the expression carrier vector of claim 8.

24. A method according to claim 23 wherein there is a T-DNA sequence at each terminus of the DNA cassette of the expression carrier vector.

25. A method according to claim 23 wherein the expression carrier vect r comprises a coding sequence within the DNA sequence cassette which encodes a dominant selectable marker for transformed plant cells.

26. The method of claim 23 wherein the plasmid of (a) is a disarmed Ti plasmid.

27. A method of transforming plant cells which comprises infecting or coculturing A. tumefaciens-susceptible plant cells with cells of A. tumefaciens which contain:

(a) a plasmid comprising the virulence region of a Ti plasmid; and

(b) the expression carrier vector of claim 11.

28. The method of claim 27 wherein the plasmid of (a) is a disarmed Ti plasmid.

29. A method according to claim 27 wherein the expression carrier vector comprises a coding sequence within the DNA sequence cassette which encodes a dominant selectable marker for transformed plant cells.

30. The method of claim 27 wherein the plasmid of (a) is a disarmed Ti plasmid.

31. A method of transforming plant cells which comprises mixing a suspension of plant cells or protoplasts thereof with an effective amount of expression carrier vector which vector includes: a promoter normally operable in plant cells and a polyadenylation signal operable in plant cells, both operably linked to a desired coding sequence.

32. A plant, including the cells and seeds thereof transformed by the method of claim 19.

33. A plant, including the cells and seeds thereof transformed by the method of claim 20.

34. A plant, including the cells and seeds thereof transformed by the method of claim 21.

35. A plant, including the cells and seeds thereof transformed by the method of claim 23.

36. A plant, including the cells and seeds thereof transformed by the method of claim 27.

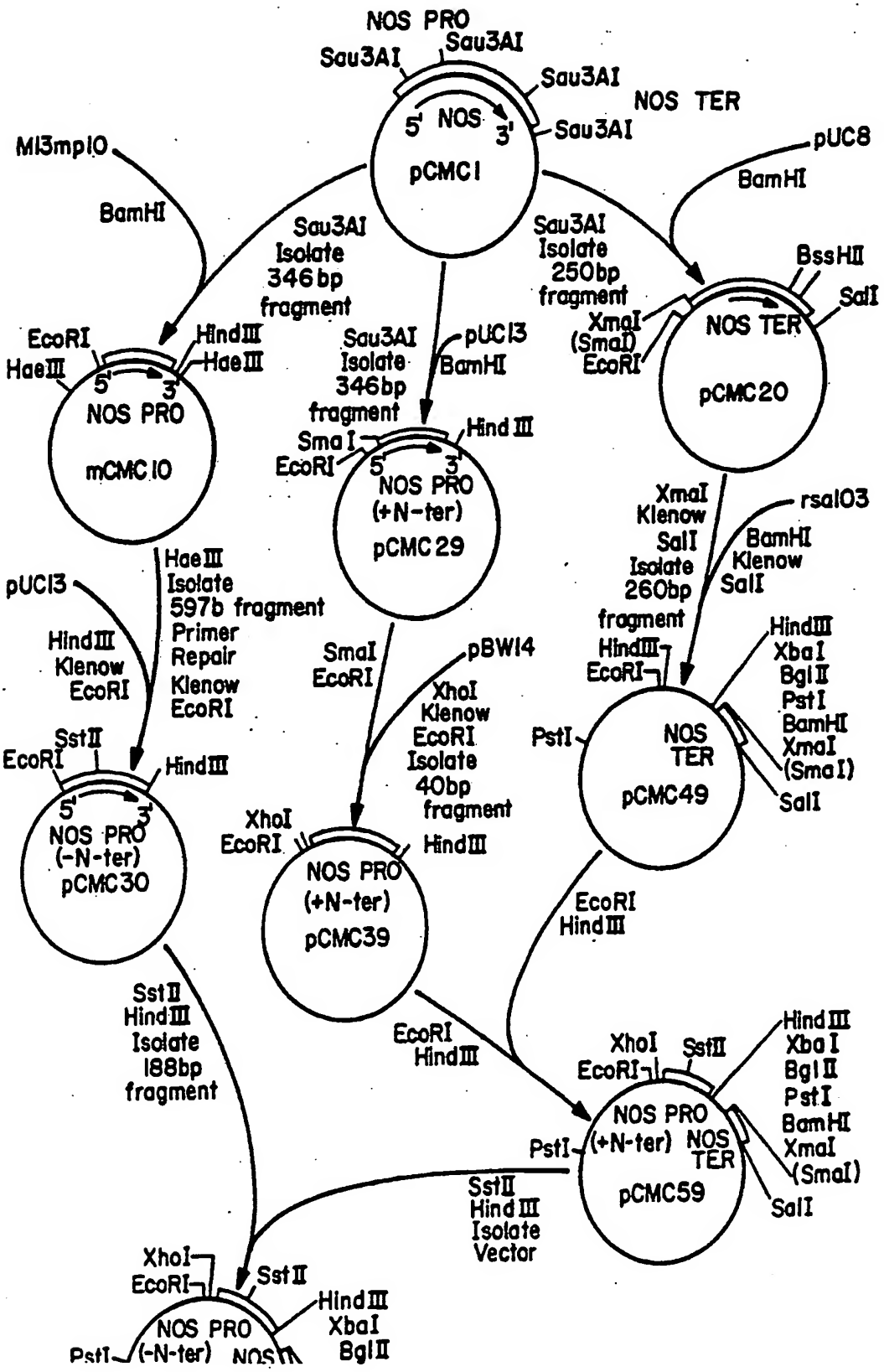
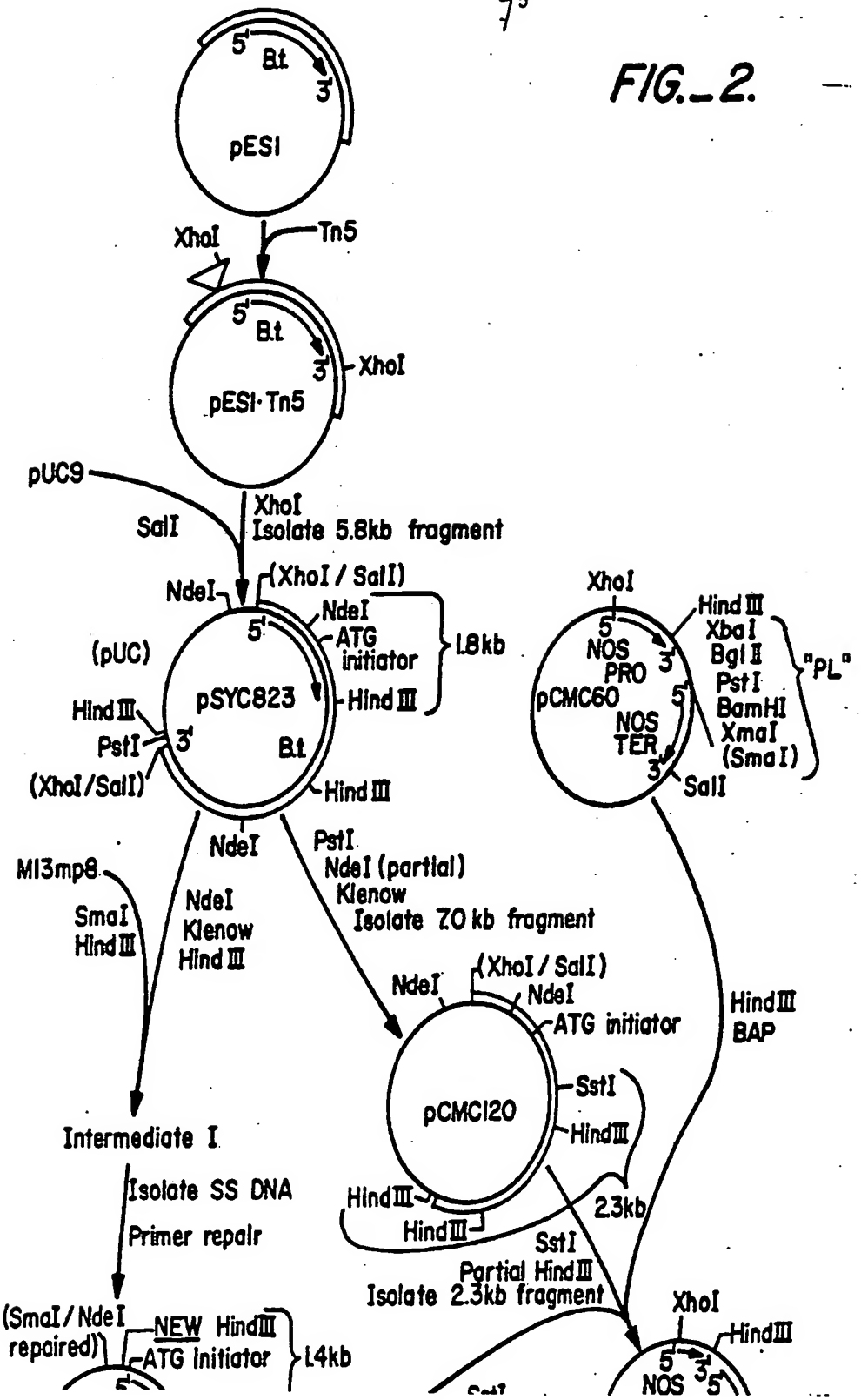

NOS PRO
Sau3AI
Sau3AI
Sau3AI
NOS TER
Sau3AI
5' NOS 3'
pCMC1
M13mp10
BamHI
pUC8
BamHI
Sau3AI
Isolate
346 bp
fragment
Sau3AI
Isolate
250bp
fragment
BssHII
SalI
XmaI
(SmaI)
EcoRI
NOS TER
pCMC20
EcoRI
HaeIII
HindIII
HaeIII
5' 3'
NOS PRO
mCMC10
Sau3AI
Isolate
346bp
fragment
pUC13
BamHI
SmaI
EcoRI
HindIII
5' 3'
NOS PRO
(+N-ter)
pCMC29
XmaI
Klenow
SalI
Isolate
260bp
fragment
rsaI03
BamHI
Klenow
SalI
HaeIII
Isolate
597b fragment
Primer
Repair
Klenow
EcoRI
pUC13
HindIII
Klenow
EcoRI
SmaI
EcoRI
pBW14
XhoI
Klenow
EcoRI
Isolate
40bp
fragment
HindIII
XbaI
BglII
PstI
BamHI
XmaI
(SmaI)
SalI
PstI
NOS
TER
pCMC49
SstII
EcoRI
HindIII
5' 3'
NOS PRO
(-N-ter)
pCMC30
XhoI
EcoRI
HindIII
NOS PRO
(+N-ter)
pCMC39
EcoRI
HindIII
SstII
HindIII
Isolate
188bp
fragment
EcoRI
HindIII
XhoI
SstII
EcoRI
HindIII
XbaI
BglII
PstI
BamHI
XmaI
(SmaI)
SalI
PstI
NOS PRO
(+N-ter) NOS
TER
pCMC59
SstII
HindIII
Isolate
Vector
XhoI
EcoRI
SstII
HindIII
XbaI
BglII
PstI
NOS PRO
(-N-ter)
NOS

FIG._2.


5'
Bt
3'
pESI
XhoI
Tn5
pESI·Tn5
pUC9
SalI
XhoI
Isolate 5.8kb fragment
(XhoI / SalI)
NdeI
ATG initiator
1.8kb
(pUC)
pSYC823
HindIII
PstI
(XhoI/SalI)
NdeI
PstI
NdeI (partial)
Klenow
Isolate 7.0 kb fragment
M13mp8
SmaI
HindIII
NdeI
Klenow
HindIII
Intermediate I
Isolate SS DNA
Primer repair
(SmaI/NdeI repaired)
NEW HindIII
ATG initiator
1.4kb
pCMC60
NOS
PRO
NOS
TER
"PL"
BglII
PstI
BamHI
XmaI
(SmaI)
pCMC120
SstI
2.3kb
Partial HindIII
Isolate 2.3kb fragment
HindIII BAP
NOS

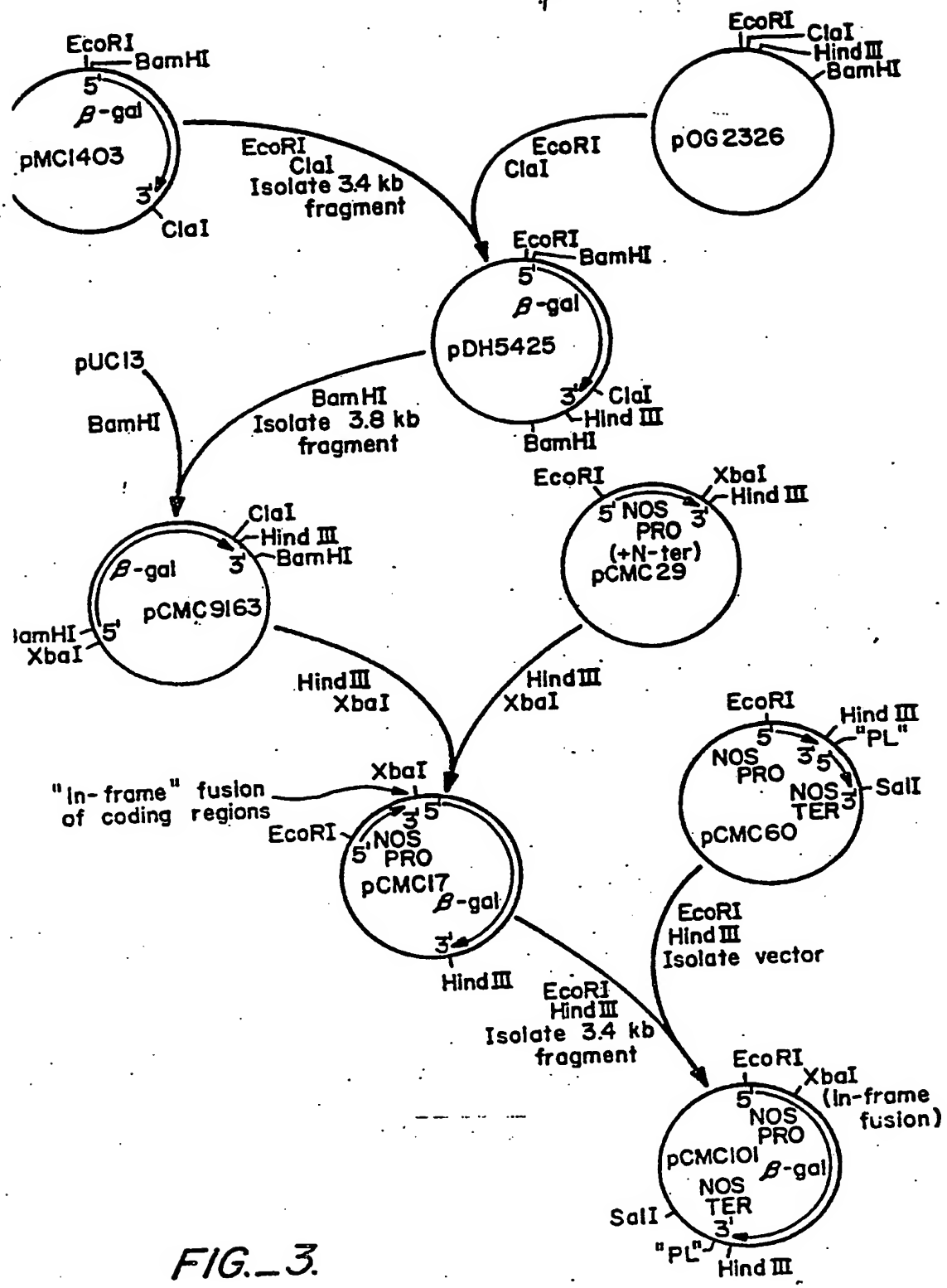

EcoRI
BamHI
5'
β-gal
pMC1403
3'
ClaI
EcoRI
ClaI
Isolate 3.4 kb
fragment
EcoRI
ClaI
Hind III
BamHI
pOG2326
EcoRI
ClaI
pDH5425
BamHI
pUC13
BamHI
BamHI
Isolate 3.8 kb
fragment
ClaI
Hind III
BamHI
pCMC9163
BamHI
XbaI
EcoRI
XbaI
Hind III
NOS
PRO
(+N-ter)
pCMC29
Hind III
XbaI
Hind III
XbaI
"In-frame" fusion
of coding regions
XbaI
EcoRI
NOS
PRO
pCMC17
β-gal
Hind III
EcoRI
Hind III
Isolate 3.4 kb
fragment
EcoRI
Hind III
"PL"
NOS
PRO
NOS
TER
SalI
pCMC60
EcoRI
Hind III
Isolate vector
EcoRI
XbaI
(In-frame
fusion)
NOS
PRO
pCMC101
β-gal
NOS
TER
SalI
"PL"
Hind III

FIG._3.

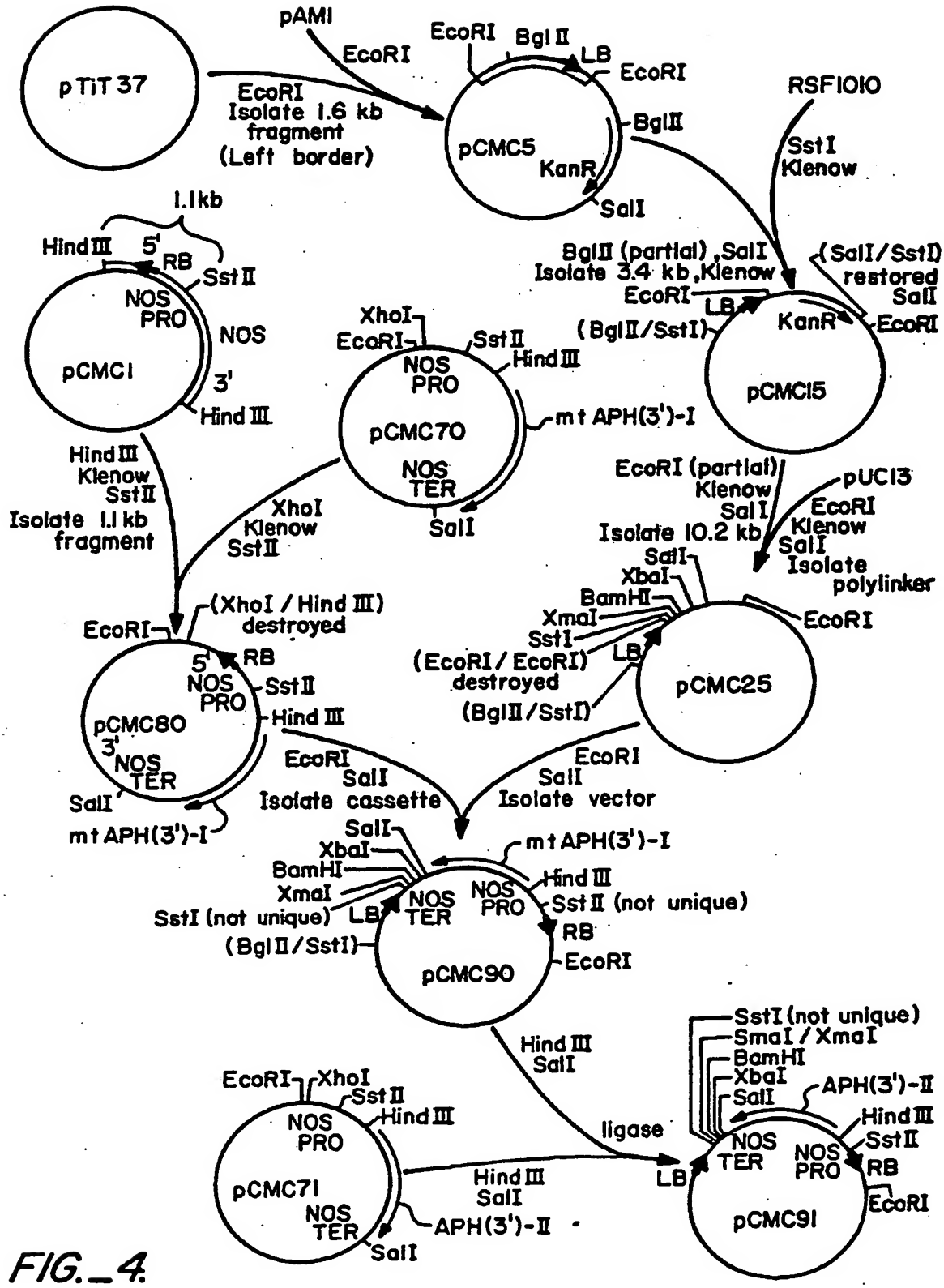

pTiT 37
pAMI
EcoRI
EcoRI
Bgl II
LB
EcoRI
EcoRI
Isolate 1.6 kb
fragment
(Left border)
pCMC5
KanR
BglII
SalI
RSF1010
SstI
Klenow
1.1kb
Hind III
5'
RB
SstII
NOS
PRO
NOS
pCMC1
3'
Hind III
BglII (partial), SalI
Isolate 3.4 kb, Klenow
EcoRI
LB
(BglII/SstI)
KanR
(SalI/SstI)
restored
SalI
EcoRI
pCMC15
XhoI
EcoRI
SstII
Hind III
NOS
PRO
pCMC70
mt APH(3')-I
NOS
TER
SalI
Hind III
Klenow
SstII
Isolate 1.1 kb
fragment
XhoI
Klenow
SstII
EcoRI (partial)
Klenow
SalI
Isolate 10.2 kb
pUC13
EcoRI
Klenow
SalI
Isolate
polylinker
SalI
XbaI
BamHI
XmaI
SstI
(EcoRI/EcoRI)
destroyed
(BglII/SstI)
LB
EcoRI
pCMC25
EcoRI
(XhoI / Hind III)
destroyed
5'
RB
NOS
PRO
SstII
Hind III
pCMC80
3'
NOS
TER
SalI
mt APH(3')-I
EcoRI
SalI
Isolate cassette
EcoRI
SalI
Isolate vector
SalI
XbaI
BamHI
XmaI
SstI (not unique)
(BglII/SstI)
mt APH(3')-I
Hind III
SstII (not unique)
RB
EcoRI
LB
NOS
TER
NOS
PRO
pCMC90
Hind III
SalI
ligase
SstI (not unique)
SmaI / XmaI
BamHI
XbaI
SalI
APH(3')-II
Hind III
SstII
RB
EcoRI
NOS
TER
NOS
PRO
LB
pCMC91
EcoRI
XhoI
SstII
Hind III
NOS
PRO
pCMC71
NOS
TER
SalI
Hind III
SalI
APH(3')-II

FIG._4.

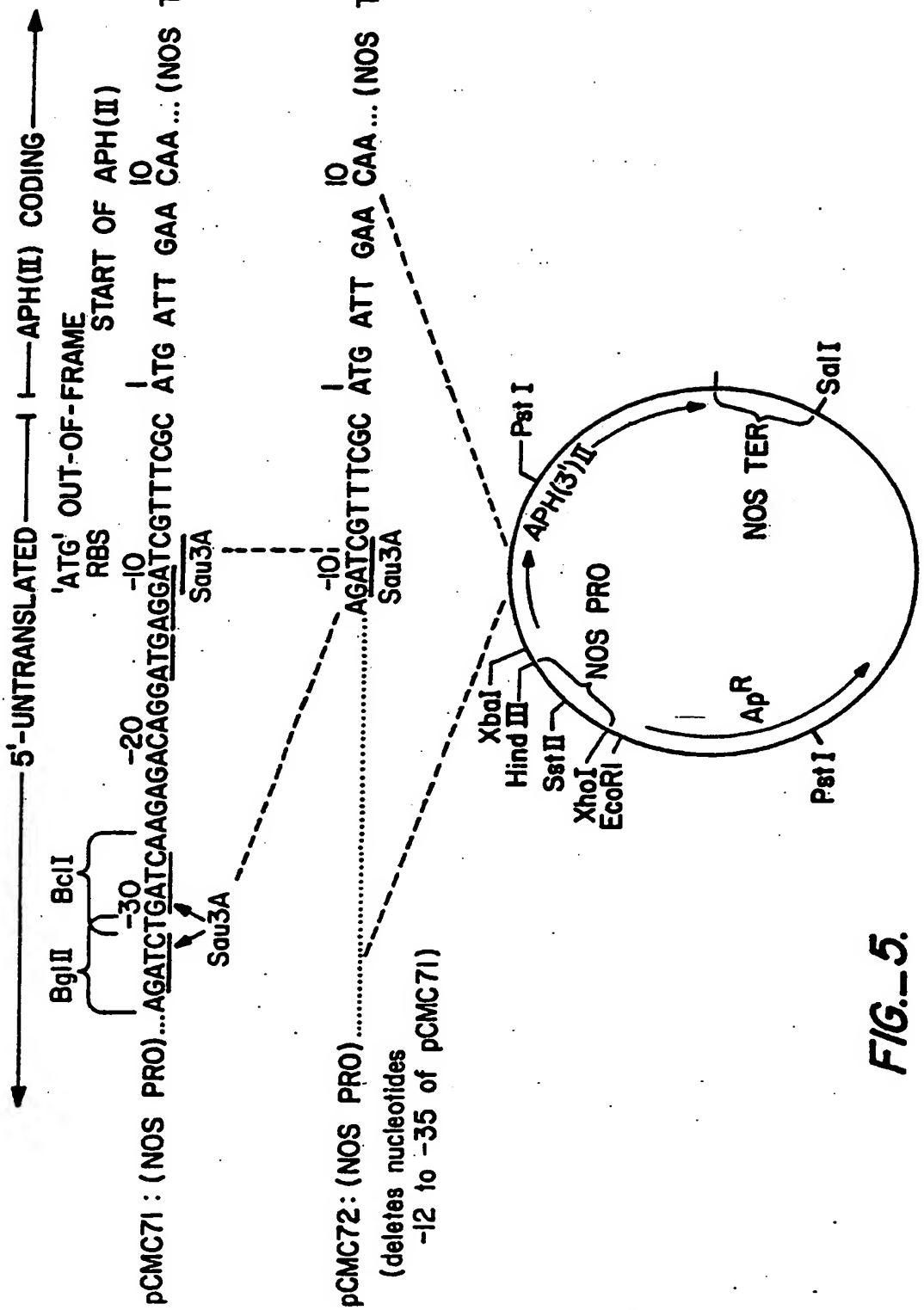

5'-UNTRANSLATED
APH(II) CODING
'ATG' OUT-OF-FRAME
RBS
START OF APH(II)
BglII BclI
-30
-20
-10
10
pCMC71 : (NOS PRO)...AGATCTGATCAAGAGACAGGATGAGGATCGTTTCGC ATG ATT GAA CAA ... (NOS TER)
Sau3A
Sau3A
pCMC72: (NOS PRO).....AGATCGTTTCGC ATG ATT GAA CAA ... (NOS TER)
-10
Sau3A
(deletes nucleotides
-12 to -35 of pCMC71)
Pst I
APH(3')II
NOS TER
Sal I
XbaI
Hind III
SstII
XhoI
EcoRI
NOS PRO
Ap^R
Pst I

FIG._5.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 85/00659

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Proceedings of the National Academy of Sciences USA, volume 80, August 1983, R.T. Fraley et al.:"Expression of bacterial genes in plant cells" pages 4803-4807; see the whole document (cited in the application)	1-14,19-31
Y	The EMBO Journal, volume 2, December 1983, P. Zambryski et al.: "Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity", pages 2143-2150; see the whole document	1-36
Y	Nature, volume 303, 19 May 1983, L. Herrera-Estrella et al.:"Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector", pages 209-213; see page 210, right hand column and page 211 (cited in the application)	1-36
A	Bio technology, volume 1, May 1983, A.J. de Framond et al.:	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th July 1985	08 AOUT 1985
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	G.L.M. Kruydenberg

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT/US 85/00659

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	"Mini-Ti: A new vector strategy for plant genetic engineering", pages 262-269; see figure 6 (cited in the application)	1-14
A	US, A, 4407956 (REGENTS OF THE UNIVERSITY OF CALIFORNIA) 4 October 1983	
P,X	Nucleic Acids Research, volume 12, November 1984, M. Bevan: "Binary Agrobacterium vectors for plant transformation", pages 8711-8721; see figure 2 and page 8717	1-14 - 19-31
P,X	WO, A, 84/2919 (MONSANTO CO.) 2 August 1984, see the claims	1-14, 19-31
P,X	EP, A, 0116718 (MAX-PLANCK-GESELLSCHAFT) 29 August 1984, see the claims	1-14, 19-31
P,X	EP, A, 0126546 (AGRIGENETICS) 28 November 1984, see claim 1	1-14

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/US 85/00659 (SA 9397)

This Annex lists the patent family memb rs relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Pat nt Office EDP file on 30/07/85

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4407956	04/10/83	None	
WO-A- 8402919	02/08/84	EP-A- 0131624	23/01/85
		JP-T- 60500438	04/04/85
EP-A- 0116718	29/08/84	AU-A- 2327484	19/07/84
		JP-A- 59140885	13/08/84
EP-A- 0126546	28/11/84	AU-A- 2684084	18/10/84

CORRECTED VERSION *

PCT

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International Bureau

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(11) International Publication Number: WO 85/ 04899

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(21) International Application Number: PCT/US85/00659

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(31) Priority Application Number: 601,904

(32) Priority Date: 19 April 1984 (19.04.84)

(33) Priority Country: US

(71) Applicant : AGRACETUS [US/US]; 8520 University Green, Middleton, WI 53562 (US).

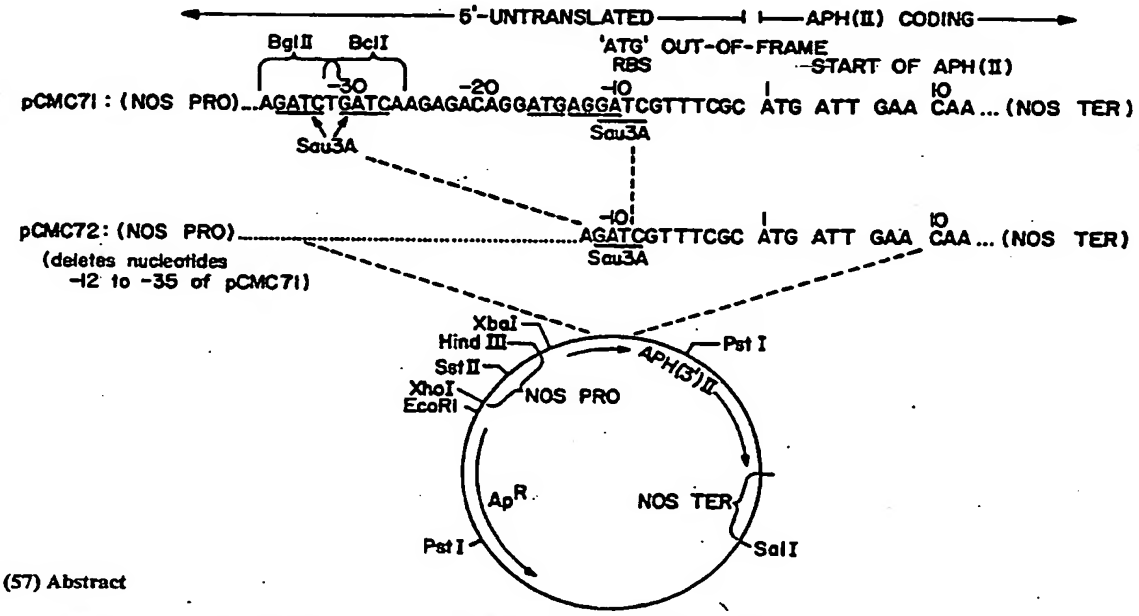
(72) Inventors: GELFAND, David, H. ; 6208 Chelton Drive, Oakland, CA 94611 (US). BARTON, Kenneth, A. ; 1718 Aurora Street, Middleton, WI 53562 (US).

(74) Agent: HALLUIN, Albert, P.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: METHODS AND VECTORS FOR TRANSFORMATION OF PLANT CELLS



(57) Abstract

Vectors and methods suitable for both direct and *Agrobacterium*-mediated transformation of plants. The vectors comprise easily manipulated expression cassettes and optionally contain border sequences associated with *Agrobacterium* plasmid DNA transfer. By using an intermediate carrier vector which contains the requisite expression control sequences in accessible form, a wide variety of single and multiple cassette vectors can be constructed.

* (Referred to in PCT Gazette No. 05/1986, Section II)